CLINICAL TRIALS IN ORGAN TRANSPLANTATION

CTOT-21

Treg Adoptive Therapy in Subclinical Inflammation in Kidney Transplantation

(TASK)

Version 11.0 / January 11, 2022

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Study Sponsor(s): The National Institute of Allergy and Infectious Diseases (NIAID)
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Protocol: CTOT-21 (TASK)	Version/Date: 11.0 / January 11, 2022
Title: Treg Adoptive Therapy in Subclinical Inflammation in Kidney Transplantation	
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INSTRUCTIONS: The site Principal Investigator should print, sign, and date at the indicated location below. A copy should be kept for your records and the original signature page sent. After signature, please return the original of this form by surface mail to:

DAIT Regulatory Management Center
PPD, Inc.
3900 Paramount Parkway
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Phone: (919) 456-4229

I confirm that I have read the above protocol in the latest version. I understand it, and I will work according to the principles of Good Clinical Practice (GCP) as described in the United States Code of Federal Regulations (CFR) – 45 CFR part 46 and 21 CFR parts 50, 56, and 312, and in the International Conference on Harmonization (ICH) document *Guidance for Industry: E6 Good Clinical Practice: Consolidated Guidance* dated April 1996. Further, I will conduct the study in keeping with local legal and regulatory requirements.

As the site Principal Investigator, I agree to carry out the study by the criteria written in the protocol and understand that no changes can be made to this protocol without the written permission of the IRB and NIAID.

_____
Site Principal Investigator (Print)

_____ _____
Site Principal Investigator (Signature) Date

Protocol Synopsis

Title	Treg Adoptive Therapy in Subclinical Inflammation in Kidney Transplantation (TASK)
Short Title	Tregs in Subclinical Inflammation
Clinicaltrials.gov Identifier	NCT02711826
Clinical Phase	Phase I/ II
Number of Sites	Cleveland Clinic Northwestern University University of Alabama at Birmingham University of California at San Francisco University of Colorado University of Michigan
IND Sponsor/Number	NIH/NIAID/DAIT/ #16626
Primary Safety Objective	This study will evaluate the safety of polyTregs in adult kidney transplant recipients.
Secondary Safety Objective	Participants receiving polyTregs will be evaluated for the safety of converting from CNI-based maintenance therapy to mTOR inhibitors after Treg therapy.
Primary Efficacy Objective	This study will evaluate whether polyclonally expanded Tregs (polyTregs) reduce graft inflammation relative to their enrollment biopsy compared to those receiving CNI-based maintenance therapy with similar baseline biopsy findings.
Secondary Efficacy Objective	This study will evaluate whether polyTregs can reduce graft inflammation by 25% or more in the 2 weeks after polyTreg infusion relative to the enrollment biopsy.
Mechanistic Objectives	This study will evaluate the impact of polyTregs on the immunological profiles of kidney transplant recipients.

Study Design	Multi-center, open-label, randomized, controlled trial with 2 cohorts: 1. Maintenance CNI based immunosuppression therapy 2. polyTregs The original study design included a third treatment arm (donor alloantigen reactive Tregs or darTregs). One subject was treated with darTregs prior to protocol version 9.0 in which this treatment arm was eliminated due to excessive manufacturing failures.
Primary Safety Endpoints	The safety of polyTregs (Group 2) will be described in comparison with CNI-based maintenance IS therapy (Group 1) by: 1. The timing and incidence of Banff 2A or higher acute cell-mediated rejection and/or acute antibody mediated rejection 2. The timing and incidence of study defined Grade 3 or higher infection
Secondary Safety Endpoints for polyTregs	The safety of PolyTregs will also be described in Groups 1 and 2 by: 1. Timing, incidence, and severity of polyTreg infusion reactions 2. Timing, incidence, and severity of culture-proven and clinically diagnosed infections after polyTreg infusion 3. Timing, incidence, and severity of acute rejection using Banff grading 4. Timing and incidence of BK viremia and CMV reactivation 5. Timing and incidence of > 10% decrease in eGFR compared to baseline
Secondary Safety Endpoint for mTOR Therapy	The safety of mTOR therapy after Treg infusion will be assessed by the incidence and timing of acute rejection. Subjects who receive Tregs and convert to mTOR therapy will be compared to subjects who did not convert to mTOR therapy, as well as subjects in Group 1.
Primary Efficacy Endpoint	The primary efficacy endpoint will be the change in inflammation as measured by the percentage area of cortex occupied by inflammatory cells using computer-assisted quantitative image analysis on the biopsy 7 months after group allocation, expressed as the percent change relative to the baseline biopsy.

Secondary Efficacy Endpoints	The secondary efficacy endpoint will be the proportion of subjects exhibiting a relative decrease of 25% or more inflammation between the baseline kidney biopsy and the biopsy 2 weeks after polyTregs, measured as the percentage area of cortex occupied by inflammatory cells using computer-assisted quantitative image analysis. Additional secondary efficacy endpoints are the number of subjects who exhibit a relative decrease of 50% or more inflammation on kidney biopsy at 2 weeks after polyTregs; and the proportion of subjects who exhibit a relative decrease of 25% or more inflammation at 7 months after group allocation.
Primary Mechanistic Endpoint	The primary mechanistic endpoints are the immunologic profiles of kidney transplant recipients using graft CRM (common response module) gene expression of rejection and/or evidence of inflammation in biopsies at 2 weeks after infusion (Group 2) and 7 months after group allocation (Groups 1 and 2).
Secondary Mechanistic Endpoints	The secondary mechanistic endpoints are the immunologic profiles of kidney transplant recipients using: 1. Persistence of infused Tregs in blood and biopsies using deuterium labeling and T cell repertoire analysis (Group 2) 2. Cytokine and CRM (common response module) mRNA and protein profiles in the urine as correlates of acute rejection and/or histologic evidence of inflammation and graft fibrosis (Groups 1 and 2) 3. Peripheral blood kSORT (kidney solid organ response test) mRNA expression of rejection/increased immune response (Groups 1 and 2)
Accrual Objective	14 Subjects Total: 7 subjects on CNI maintenance therapy 7 subjects to receive polyTregs
Study Duration	6.5 Year Accrual, 12 Months Follow Up
Treatment Description	Subjects will be on maintenance IS therapy using CNI + MMF/MPA with or without steroids at the time of study entry. Eligible subjects will be randomized to receive: 1. Standard CNI maintenance IS (no Tregs) 2. 550 ± 450x 10^6 polyTregs After receiving at least 300 x 10^6 polyTregs infusion, eligible subjects will start mTOR inhibitor.

Inclusion Criteria	Individuals who meet all of the following criteria are eligible for enrollment as study participants: 1. Subject must be able to understand and provide informed consent 2. Age ≥18 years of age at the time of study entry 3. Recipients of non-HLA identical living or deceased donor renal transplants 4. Protocol renal allograft biopsy at 5 months (± 8 weeks) after transplantation with Banff i1 and/or ti1 with concomitant t scores t0, t1, t2 or t3; Banff i2 and/or ti2 with concomitant t scores t0 or t1; and without v > 0, [ptc + g] ≥2, C4d >1 (by IF), or C4d > 0 (by IHC) confirmed by central pathologist. Subjects must not be treated for pathologic criteria (e.g., steroids). 5. eGFR ≥30 ml/min at the time of study entry 6. Maintenance immunosuppression consisting of tacrolimus, MMF/MPA ± prednisone (≤10 mg/day) 7. Current immunizations including TdAP, pneumococcal and seasonal influenza vaccines prior to study treatment, completed prior to randomization and no less than 14 days prior to planned manufacturing collection 8. Hepatitis B serologies must be: a. Positive HB surface antibody, negative HB core antibody and negative HB surface antigen for recipients immune to hepatitis B b. Negative HB surface antibody, negative HB core antibody and negative HB surface antigen for non-immune/ HBV naïve recipients provided donor had negative HB core antibody and negative HB surface antigen at the time of donation 9. Negative TB test (PPD, interferon-gamma release assay, ELISPOT testing) within 1 year prior to enrollment. Subjects with a history of TB (positive TB test without active infection) must have completed one of the latent TB infection treatment regimens endorsed by the CDC (Division of TB Elimination, 2016). Alternative regimens for latent TB infection eradication will be adjudicated by the site's infectious disease specialist. 10. Female subjects with childbearing potential must have reviewed Mycophenolate REMS and have a negative pregnancy test upon study entry. 11. Female subjects with childbearing potential must agree to use FDA approved methods of birth control for the duration of the study; subjects must consult with their physician and determine the most suitable method(s) that are greater than 80% effective (http://www.fda.gov/birthcontrol)

Exclusion Criteria	Individuals who meet any of these criteria are not eligible for enrollment as study participants: 1. Inability or unwillingness of a participant to give written informed consent or comply with study protocol 2. History of malignancy; except adequately treated basal cell carcinoma 3. History of graft loss from acute rejection within 1 year after any previous transplant 4. History of transplant renal artery stenosis 5. History of cellular rejection prior to enrollment that did not respond to steroids and/or subsequent creatinine after treatment for rejection greater than 15% above baseline 6. Known hypersensitivity to mTOR inhibitors or contraindication to everolimus (such as history of wound healing complications) 7. Any chronic illness requiring uninterrupted anti-coagulation after kidney transplantation 8. Post-transplant DSA >5000 MFI or post-transplant treatment with IVIg for DSA. Enrolled subjects with post-transplant DSA >2000 MFI will not be eligible for mTOR conversion. 9. Positive HIV 1 or HIV 2 serology prior to transplantation 10. Known positive HBSAg, or HBcAb serology 11. Proteinuria with urine pr/cr > 1.0 g/g 12. Any condition requiring chronic use of corticosteroids >10mg/day at the time of study entry 13. Subjects requiring treatment for pathologic findings on study eligibility biopsy (see inclusion 4). 14. Active infection at the time of study entry 15. History of active TB or latent TB without adequate treatment (see inclusion 10). 16. Serum BK virus >1,000 copies/ml by PCR at the time of study entry 17. Hematocrit <27%; ANC < 1,000/µL; lymphocytes <500/µL; at the time of study entry 18. Participation in any other studies with investigational drugs or regimens in the preceding year 19. Any condition or prior treatment which, in the opinion of the investigator, precludes study participation 20. Unable to provide adequate biopsy specimen (paraffin embedded formalin fixed) from eligibility biopsy (3-7 months post-transplant) for quantitative analysis. 21. EBV naïve recipient of a kidney from an EBV positive donor, historically EBV naïve recipient with primary EBV infection at the time of screening (primary anti-VCA IgM, without antibody to EBNA), positive EBV PCR 22. Hepatitis C Virus AB positive subjects with negative HCV PCR are eligible if they have spontaneously cleared infection or are in sustained virologic remission for at least 12 weeks after treatment. 23. Positive SARS-CoV2 testing by RT-PCR

Treg Infusion Inclusion Criteria	1. Individuals randomized to group 2 who continue to meet all of the enrollment criteria are eligible for Treg infusion. 2. Negative SARS-COV2 RTPCR testing within 1 week of Treg infusion
Treg Infusion Exclusion Criteria	1. Received any vaccination within 14 days prior to blood collection for Treg manufacture 2. Unacceptable Treg product. 3. Positive pregnancy test for women of childbearing potential.
mTOR Conversion Inclusion Criteria	1. Received at least 300 x 10^6 polyTreg infusion 2. Resolution of inflammation on the 2-week post-infusion biopsy as compared to the baseline biopsy, confirmed by central pathologist
mTOR Conversion Exclusion Criterion	1. Post-transplant DSA >2000 MFI 2. Any condition or clinical variable, which in the opinion of the site investigator, precludes conversion to mTOR.

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TUBULAR EPITHELIAL INJURY AND RENAL FIBROSIS BIOMARKERS

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Table of Contents

Glossary of Abbreviations

CFR	Code of Federal Regulations
COVID-19	Coronavirus Disease 2019
CRF	Case Report Form
CRM	Common Response Module
CTCAE	Common Terminology Criteria for Adverse Events
DAIT	Division of Allergy, Immunology, and Transplantation
darTregs	Donor Alloantigen Reactive T Regulatory Cells
DSMB	Data Safety Monitoring Board
FDA	Food and Drug Administration
GCP	Good Clinical Practice
ICH	International Conference on Harmonization
IND	Investigational New Drug
IRB	Institutional Review Board
kSORT	Kidney Solid Organ Response Test
mTOR	Mammalian Target of Rapamycin
NIAID	National Institute of Allergy and Infectious Diseases
PI	Principal Investigator
polyTregs	Polyclonal T Regulatory Cells
Mycophenolate REMS	Mycophenolate Risk Evaluation and Mitigation Strategy
SAE	Serious Adverse Event
SAP	Statistical Analysis Plan
SAR	Suspected Adverse Reaction
SARS-CoV2	Severe Acute Respiratory Syndrome Coronavirus 2
SCI	Subclinical Inflammation
SOP	Standard Operating Procedure
SUSAR	Serious Unexpected Suspected Adverse Reaction

Study Definitions Page

Acute Cell-mediated Rejection	Banff 2007 Type 1A or higher and clinical treatment for acute rejection. Central reading will be utilized when accounting for study stopping rule and for safety endpoint.
Severe Acute Cell-mediated Rejection	Banff 2007 Type 2A or higher and clinical treatment for acute rejection. Central reading will be utilized when accounting for study stopping rule and for safety endpoint.
Antibody Mediated Rejection	Diffusely positive staining for C4d, presence of circulating anti-donor antibodies, and morphologic evidence of acute tissue injury.
Graft Inflammation	Banff i1 and/or ti1+ t0, t1, t2 or-t3 or Banff i2 and/or ti2 + t0 or t1
Graft Failure	90 consecutive days of dialysis dependency
Infection, Severe	Study defined grade 3 or higher; culture-proven and clinically diagnosed
Investigational Agent	polyTregs, everolimus
Protocol Mandated Procedures	Any procedure or specimen collection performed solely for the purpose of this research study, not considered site specific standard of care.
Randomized	A subject who met eligibility criteria, signed informed consent document, and was randomly assigned to one of two treatment groups.
Withdrawn from Study Therapy	Subjects who prematurely discontinue the study after receiving polyTregs.
Treatment for Rejection	Treatment of rejection in this protocol constitutes use of IV steroids and/or Thymoglobulin. Adjustment of maintenance immunosuppression medications will not be counted as treatment for rejection.
Women of Childbearing Potential	WOCBP includes any female who has experienced menarche and who has not undergone successful surgical sterilization (hysterectomy, bilateral tubal ligation, or bilateral oophorectomy) or is not postmenopausal (defined as amenorrhea ≥ 12 consecutive months; or women on hormone replacement therapy with documented serum follicle stimulating hormone level > 35 mIU/mL). Even women who are using oral, implanted, or injectable contraceptive hormones or mechanical products such as an intrauterine device or barrier methods (diaphragm, condoms, spermicides) to prevent pregnancy or practicing abstinence or where the partner is sterile (e.g., vasectomy), should be considered to be of childbearing potential.

1. Study Hypotheses/Objectives

1.1. Hypotheses

This study will explore the following hypotheses:

1. PolyTreg infusions are safe and will increase the absolute number of Tregs in circulation and in the kidney allograft.
2. PolyTregs will suppress inflammation in the graft and reduce markers of inflammation in the graft and urine

1.2. Primary Safety Objective

This study will evaluate the safety of polyTregs in adult kidney transplant recipients. Incidence of death, graft loss, acute rejection and severe infections will be compared and overall safety events, including infusion reactions, will be described.

1.3. Secondary Safety Objective for mTOR Therapy

Participants receiving polyTregs will be evaluated for the safety of converting from CNI-based maintenance therapy to mTOR inhibitors after Treg therapy.

1.4. Primary Efficacy Objective

This study will evaluate whether polyTregs reduce graft inflammation relative to their enrollment biopsy compared to those receiving CNI-based maintenance therapy with similar baseline biopsy findings.

1.5. Secondary Efficacy Objective

This study will evaluate whether polyTregs can reduce graft inflammation by 25% or more in the 2 weeks after the polyTreg infusion relative to the enrollment biopsy.

1.6. Mechanistic Objective

This study will evaluate the impact of polyTregs on the immunological profiles of kidney transplant recipients.

2. Background and Rationale

2.1. Background and Scientific Rationale

2.1.1 Chronic Allograft Dysfunction

Despite advances in transplantation reducing early acute rejection rates to <15% and improving 1-year graft survival to >90%, long-term graft attrition rates have remained unchanged at 4% loss per year (Meier-Kriesche HU, 2004). A major contributor to graft loss is progression of interstitial fibrosis and tubular atrophy (IF/TA) (Nankivell BJ F.-L. C., 2001) (Cosio FG, 2005) (Nankivell BJ B. R., 2003), which is the result of cumulative allograft damage of immunologic and nonimmunologic origin. Studies of protocol biopsies have provided important clues on how early changes in graft histology relate to long-term graft survival. Early sequential post-transplant protocol biopsies show a rapid increase in the prevalence of IF/TA (Cosio, 2005). When separated into two categories: IF/TA with and IF/TA without inflammation, it appears that is not IF/TA per se that is detrimental, but IF/TA associated with and/or secondary to inflammation that results in progression of renal dysfunction (Park, 2010) (Mannon, 2010). Chronic immune injury in the allograft is now recognized in the majority of patients with late graft loss (Mannon, 2010) (El-Zoghby ZM, 2009).

2.1.2 Graft Inflammation

Inflammation manifested as infiltration of mononuclear cells in the renal allograft appears to be detrimental to the graft in the long term, even when insufficient to meet Banff criteria for acute cellular rejection (types I, II or III). According to the Banff classification, interstitial inflammation (i1, i2 and i3) without tubulitis (t0) is considered “nonspecific”. When tubulitis is present but mild (t1) or accompanied by subthreshold inflammation (t2 with i0-i1), it is termed “borderline change” and deemed “suspicious for acute rejection” (Racusen LC, 1999). Such changes are common, reported in 11-44% of surveillance biopsies within the first year (Gloor JM, 2002) (Heilman RL, 2010) (Thierry A, 2011). In one study, biopsies with borderline change had an intermediate level of expression of proinflammatory genes between that of histologically normal biopsies and those with acute rejection (Hoffmann SC, 2005). Another study found that a transcriptional profile indicative of activated Th1 cells, but not the magnitude or composition of the infiltrate, uniquely defined a functionally significant allograft rejection (Lipman ML, 1998). Thus, it appears that the immune response is qualitatively similar but quantitatively reduced in SCI and borderline change, indicating that these entities carry the same potential for alloimmune damage as clinical acute rejection. A study of 124 sequential protocol biopsies in 46 patients who exhibited histologic evidence of chronic allograft nephropathy at 1 year found that the presence of subclinical rejection correlated with histologic progression of chronicity, a lower creatinine clearance at 5 years, and worse long-term graft survival (Shishido S, 2003). In another study of 435 allograft recipients, the combination of SCI injury and IF/TA on protocol biopsies was strongly associated with later graft failure, more so that IF/TA alone (Moreso F, 2006). Similarly, in 292 recipients both of living and deceased donor grafts, inflammation even with mild fibrosis predicted worsening graft function (Cosio FG, 2005). In contrast, the absence of acute inflammation at any time point was associated with minimal deterioration in renal function or progression of renal lesions. Finally, a study of 151 living-donor recipients found that the combination of fibrosis and inflammation in 1-year protocol biopsies was associated with a rejection-like gene expression signature, reduced graft function, and shortened graft survival, even among tacrolimus + MPA-treated recipients without overt risk factors for poor outcomes (Park WD, 2010). In these patients, the iothalamate clearance at 3 years in those with inflammation and fibrosis was 52 ± 18 ml/min vs. 70 ± 23 ml/min in those with normal histology (p=0.01). Increased expression of innate and adaptive immune transcripts were seen in the group with inflammation and fibrosis, suggesting that early interventions aimed at altering rejection like inflammation may succeed in stabilizing graft function and extending graft survival. Also, while the previous discussion has highlighted the role of inflammation in areas of viable cortex of the kidney, recent studies demonstrate that inflammation in areas of fibrosis and atrophy, not usually scored by Banff criteria, are strongly associated with later graft loss (Mannon RB M. A.,

2010)(Mengel M, 2009). Efforts to abrogate these infiltrates as a means of mitigating graft dysfunction have not been studied.

2.1.2.1 Incidence and Impact of Sub-Clinical Inflammation

A retrospective analysis of 380 consecutive 6-month surveillance kidney transplant biopsies at UCSF Medical Center between 7/2009-5/2011 scored by a single pathologist using the revised Banff 2007 classification (Solez K, 2008) found subclinical borderline change as defined by Banff, in 20.3%, and inflammation (Banff i1-i2) with minor or no tubulitis (t0 or t1) in 10.9% of all biopsies without acute rejection (Table 1).

Table 1. 6-month protocol biopsies* at UCSF sorted by inflammation (i) and tubulitis (t) scores

Banff Category	t0	t1	t2	t3	Total
i0	261 (68.7%)	41 (10.8%)	1 (0.3%)	0	303 (79.7%)
i1	9 (2.4%)	23 (6.1%)	2 (0.5%)	0	34 (8.9%)
i2	1 (0.3%	8 (2.1%)	0	0	8 (2.4%)
i3	0	3 (0.8%)	0	0	3 (0.8%)
Total	271 (71.3%)	75 (9.7%)	3 (0.8%)	0	349 (91.8%)

*Note: Of a total of 380 six-month biopsies, 31 had acute rejection meeting Banff criteria. Numbers in parentheses are percentages of all 380 biopsies.

In this analysis, chronic graft injury was significantly associated with the presence of inflammation. Banff ci-scores ≥1 (representing interstitial fibrosis) and ct-scores ≥1 (representing tubular atrophy) were significantly more common in biopsies with inflammation than those without (62% vs. 14% for ci-score, p-value <0.0001; 78% vs. 53% for ct-score, p-value <0.0001). Additionally, inflammation was associated with lower eGFR by the Modification of Diet in Renal Disease (MDRD) equation (Levey AS, 1999)at 6 months (60 vs. 67 ml/min/1.73 m2, p-value <0.0001) and at 12 months (58 vs. 68 ml/min/1.73 m2, p-value <0.0001). Our findings are very similar to those seen in a study of 151 low risk, living-donor kidney transplant recipients on a CNI-based regimen (Park WD, 2010). In this study, 13.2% of 1-year protocol biopsies showed fibrosis with inflammation and these patients on follow-up exhibited a decline in GFR and reduced graft survival.

In our cohort, a follow up biopsy was performed 6 months later in 23 recipients who were found to have subclinical rejection or borderline change on their 6-month protocol biopsy. At 12 months, persistent inflammation was seen in over 56.5% despite treatment, which included methylprednisolone pulse, Thymoglobulin and IVIg for subclinical rejection and oral prednisone pulse for those with borderline change. Therefore, it appears that SCI, which has been demonstrated to be damaging to the graft in the long-term, may persist despite conventional therapy and potentially lead to a long-term decline in graft function.

2.1.2.2 Rationale and Experience with Multiplexed Immunofluorescence and in situ Hybridization for Detection of Graft Inflammation

The Banff classification (Banff in brief), based on semiquantitative assessment of morphologic changes including inflammation, is considered the “gold standard” for evaluation of kidney transplant biopsies. However, the lack of qualitative information on the composition of the inflammation, imprecise quantification, and poor inter-observer reproducibility, make Banff a suboptimal tool for research. Since precise quantitative assessment of the inflammation in our study is essential to monitor treatment-related changes in follow-up biopsies, we have developed a quantitative immunofluorescence (IF) assay in formalin-fixed paraffin-embedded (FFPE) tissues combined with whole slide digital imaging and computer-assisted image analysis to measure the inflammatory load. The application of novel multiplex IF technologies also allows us to perform a detailed qualitative assessment of the inflammation in the biopsies.

Multiplex immunofluorescence (IF) assays to measure inflammation quantitatively and qualitatively: To take advantage of better morphology of FFPE tissues and easy multiplexing capabilities of IF, we developed various sets of IF stains on FFPE kidney biopsies using a combination of inflammatory cell markers to measure inflammation (1) quantitatively and (2) qualitatively. The total inflammatory load is measured by using leukocyte common antigen (LCA) as a marker while the composition of the inflammation is assessed via multiplexing including CD8, CD4, FoxP3, CD20, and CD68 markers, among others. Validation of the LCA assay revealed excellent reproducibility and repeatability. Twenty-eight serially cut sections of transplant kidney biopsies were stained with LCA. Whole slide digital images were generated from each section and the signals were quantitated in each section. The average values from the corresponding consecutive sections stained by one operator versus those stained by another operator were statistically analyzed. No significant differences in LCA expression were noted in any of the corresponding sections.

Multiplex IF and in situ hybridization (miFish) stains on FFPE tissues: Immunohistochemistry (IH)/IF is often hampered by subpar sensitivity and specificity of certain antibodies that are difficult to validate. In some cases, the low-level expression of antigens, such as seen with certain cytokines, makes immunohistochemical detection of these antigens difficult or impossible. As an alternative to IH/IF, we have adapted the RNAScope® in situ hybridization (ISH) platform to analyze FFPE biopsies for mRNA expression. This is a novel and highly sensitive ISH technology with very high specificity. To determine the cellular source of mRNA (IL-6) expression, we applied multiplex IF stains, similar to that described above, to the same sections that the ISH was performed on. We conclude that multiplex IF stains can be performed following ISH hybridization. The quality of both the ISH and multiplex IF signals is sufficient to apply computer-assisted image analysis tools for quantitation

2.2. Rationale for Selection of Investigational Product or Intervention

2.2.1. Treg Manufacturing

UCSF has developed a GMP-compliant process for producing polyTregs (Putnam, 2009). This process starts by purifying $CD4^+CD25^+CD127^{lo}$ Tregs from peripheral blood or leukapheresis product using single–step fluorescence activated cell sorting (FACS) for $CD4^+CD25^+CD127^{lo}$ cells or sequential magnetic activated cell sorting (MACS) to enrich for $CD25^+$ cells followed by FACS purification of $CD4^+CD127^{lo}$ cells. The purified Tregs are stimulated twice with anti-CD3 and anti-CD28 beads on day 0 and day 9, leading to 300 to 3000-fold expansions in a 14-day period. Up to 5 billion polyTregs can be produced from one unit of blood. The expanded Tregs retain their phenotype and are highly suppressive in vitro. Tregs manufactured using this protocol have been infused into 14 type 1 diabetes patients in an UCSF-led trial. To determine if this process can be used to expand Tregs from immunosuppressed kidney transplant patients, we have recently expanded Tregs isolated from transplant recipients receiving tacrolimus and MPA. As shown in Figure 1, Tregs from this immunosuppressed patient expanded comparably to Tregs from non-transplant patients who were not receiving immunosuppressive therapy. Post expansion flow cytometric analysis shows that the expanded CD4+ T cells are uniformly CD25+ and FOXP3+. Thus, it is feasible to expand polyTregs from patients on immunosuppression.

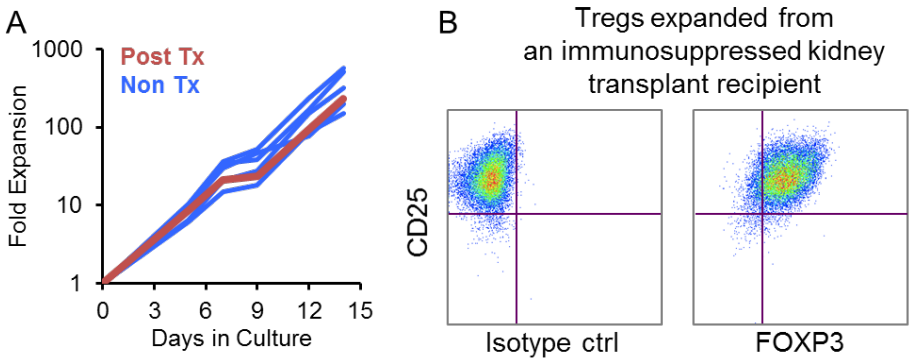


Figure 1: Expansion of Tregs isolated from a transplant recipient on immunosuppression. A. Expansion curves of Tregs isolated from an immunosuppressed transplant recipient (red line) and non-immunosuppressed patients (blue lines). **B.** Post expansion analysis of Tregs expanded from transplant recipient showing expression of FOXP3 and CD25 on CD4+ T cells.

Figure 1. Expansion of Tregs isolated from a transplant recipient on IS

2.3. Preclinical Experience

Tregs are a small subset of CD4+ T cells that depend on the FOXP3 transcription factor for their lineage differentiation and function. They function by preventing the initiation of unwanted immune activation and by suppressing ongoing immune responses to limit bystander tissue destruction. Thus, Tregs are a natural part of an immune response, essential for resolving the associated inflammation. In kidney transplantation, acute rejections as well as borderline change are often associated with an increase of Tregs in the graft (Muthukumar T, 2005) (Taflin C N. D., 2010) and it has been suggested that Treg recruitment at the acute phase of the allogeneic response can diminish the interstitial inflammation and reverse the rejection. Thus, infusion of Tregs before extensive graft damage may improve long-term graft outcomes (Bluestone, 2004) (Kang, 2007) (Long, 2009) (Sagoo, 2008) (Waldmann, 2008) (Walsh, 2004) (Wood, 2003). Unlike generalized immunosuppressive regimens, Tregs are long-lived and can function in a dominant and antigen specific manner. Thus, therapeutic infusion of Tregs has the potential to induce long-term donor-specific tolerance without impeding desired immune responses to pathogens and tumors in transplant patients.

Preclinical studies have demonstrated that Tregs can be used to control alloimmune responses in graft-versus host-disease (GvHD) as well as organ and cell transplantation models. Treg reactivity, dosing, adjunct immunosuppression, and timing of Treg infusion critically impact the efficacy of Treg therapy in organ transplantation (Wood, 2003). Tregs enriched for donor alloantigen reactivity are more effective than polyclonal Tregs. Most mouse experiments have evaluated Treg therapy without adjunct immunosuppression and administered Tregs at the time of transplantation. However, the peri-transplant period is not the ideal time for Treg therapy because the high intensity immunosuppression used at this time may antagonize Tregs and inflammation triggered by surgical trauma and ischemia reperfusion may destabilize Tregs. In addition, the complexity of the peri-transplant period may render it difficult to assess the safety profile of Treg therapy. Therefore, we favor the use of Tregs when patients are more stable at least several months after transplant. Results from mouse models show that delayed administration of Tregs is effective at inducing long-term drug-free graft survival when the recipients are initially treated with depleting agents or mTOR inhibitors (Fan Z, 2010) (Raimondi, 2010).

2.4. Clinical Studies

A key advance for Treg therapy in humans is the finding that human Tregs can be isolated and expanded in vitro while maintaining their immunoregulatory function. As of mid-2013, 1 case study and 2 clinical trials evaluating the safety and efficacy of Tregs in treating GvHD have been reported, all demonstrating promising safety and potential efficacy profiles (Brunstein, 2011) (Di Ianni, 2011) (Trzonkowski, 2009). These studies treated bone marrow transplant recipients with Tregs obtained from the bone marrow or third-party donors. A total of 53 patients were treated with Treg doses ranging from 3 to 6 x 10^6 Tregs/kg body weight. A more recent trial infused 10-20 x 10^6/kg ex vivo expanded $CD3^+CD4^+CD25hiCD127-$ Tregs in children with recent onset type 1 diabetes and found the treatment well tolerated and associated with preservation of C-peptide 6 months after the infusion (Marek-Trzonkowska N, 2012). In a study in type 1 diabetes at UCSF, Bluestone and colleagues have performed a dose escalation study treating 14 patients. Patients showed no signs of a serious safety signal. Persistence of infused Tregs at 6 months after infusion; and preservation of C-peptide as long as 2 years after infusion have been observed.

2.4.1. Rationale for Timing of Treg Infusion

The choice of introducing the Tregs 3-7 months after transplantation offers multiple advantages over therapy at the time of surgery or early after transplantation. By delaying the administration of Tregs, we avoid the effect of induction therapy (especially the clearly detrimental effect of the anti-IL-2R antibodies) and the high exposure of CNIs that is required early post-transplant. The choice of using patients with inflammation will allow direct assessment of impact of Tregs on the graft in the follow up biopsy at 2 weeks and at 6 months after the therapy. In addition, it is safer to convert to an mTOR inhibitor to provide Tregs the optimum environment for in vivo expansion and durability later post-transplant.

2.4.2. Rationale for Dose Selection

The Treg dose was chosen by considering three primary factors: estimated effective dose, previous experiences in human patients, and manufacturing capacity.

The effective dose of Treg therapy for reversing inflammation in allografts is currently unknown. Based on preclinical studies of Treg therapy in transplantation, we estimate that inducing transplant tolerance would require more than 50 x 10^9 of polyTregs. This dose may be reduced by an order of magnitude by depleting T cells, using Treg-supportive immunosuppression, or the avoidance of the highly inflammatory conditions during the period immediately following the transplant (Tang Q, 2012). Therefore, we estimate that 5 x 10^9 polyTregs will likely to have an impact of the anti-graft response.

Among all the published studies of Treg therapy in humans, the highest dose infused is 6 x 10^6/kg polyTregs in adults for GvHD prevention (Di Ianni, 2011) and 20 x 10^6/Kg polyTregs in children with type 1 diabetes (Marek-Trzonkowska N, 2012). A phase 1 polyTreg therapy trial completed enrollment and infusion in October 2013 at UCSF. A total of 14 patients with recent onset of type 1 diabetes were infused in the highest dosing cohort receiving 2.6 x 10^9 total Tregs (equivalent to 37 x 10^6/kg).

As of January 2017, three kidney transplant recipients with subclinical inflammation 6 to 7 months after transplant were enrolled in a pilot trial and received 320 x 10^6 polyTregs at UCSF. All patients have completed 1 year of follow up after the infusion of polyTregs. None of the patients had an infusion reaction. The first patient experienced transient 50% reduction of all leukocyte counts that began 4 days after Treg infusion and resolved by 28 days after infusion. The patient was completely asymptomatic during this period. The protocol biopsy taken 2 weeks after Treg infusion showed complete resolution of the subclinical inflammation. It is unclear whether leukopenia and clearance of inflammation is

related to polyTregs infusion in this patient. Leukopenia was not observed in any of the type 1 diabetes patients that received up to 2.6 x 10^9 polyTregs manufactured in the same facility. However, it is possible that 320 x 10^6 polyTregs of polyTregs may have a biological impact when given to patients who are also on triple immunosuppression regimen consisted of CNI, MMF, and steroids. Of note, leukopenia was not seen in the 2nd and 3rd patients in this trial. None of the patients experienced any treatment related serious AEs.

We plan to infuse 550 ± 450 x 10^6 of polyTregs to patients enrolled in this trial. This dose is higher than targeted earlier in this protocol. At the time when this protocol was first implemented, we planned to compare the efficacy of polyclonal vs donor alloreactive Tregs and wished to keep Treg dose the same between the two arms in order to make a rigorous comparison. Given that the donor alloreactive Treg arm has been discontinued, we do not have any reason to limit the target dose in the polyTreg arm; especially since a higher dose is expected to have more biologic activity (Battaglia M., 2005). We have chosen a target dose of 550 ± 450 x 10^6 polyTregs because it is within the range that has been safely infused in humans (Bluestone J., 2015), is highly feasible to produce, and provides the best chance of efficacy.

2.4.3. Rationale for Everolimus

The mechanistic target of rapamycin (mTOR) pathway integrates diverse environmental inputs, including immune signals and metabolic cues, to direct T cell fate decisions (H, 2012). In the Treg compartment, the Akt-mTOR axis is widely acknowledged as a crucial negative regulator of Treg *de novo* differentiation (Haxhinasto S, 2008) (Sauer S, 2008) (Liu G, 2009) (Liu G Y. K., 2010) and population expansion (Battaglia M, 2005). In contrast to CNIs, sirolimus, an mTOR inhibitor, has been shown to favor Treg expansion and survival both *in vitro* (Strauss L, 2009) (Gallon L, 2015) (Bocian K, 2010) (Levitsky J, 2011)and in vivo (Battaglia M S. A.-H., 2006) (Akimova T, 2012)

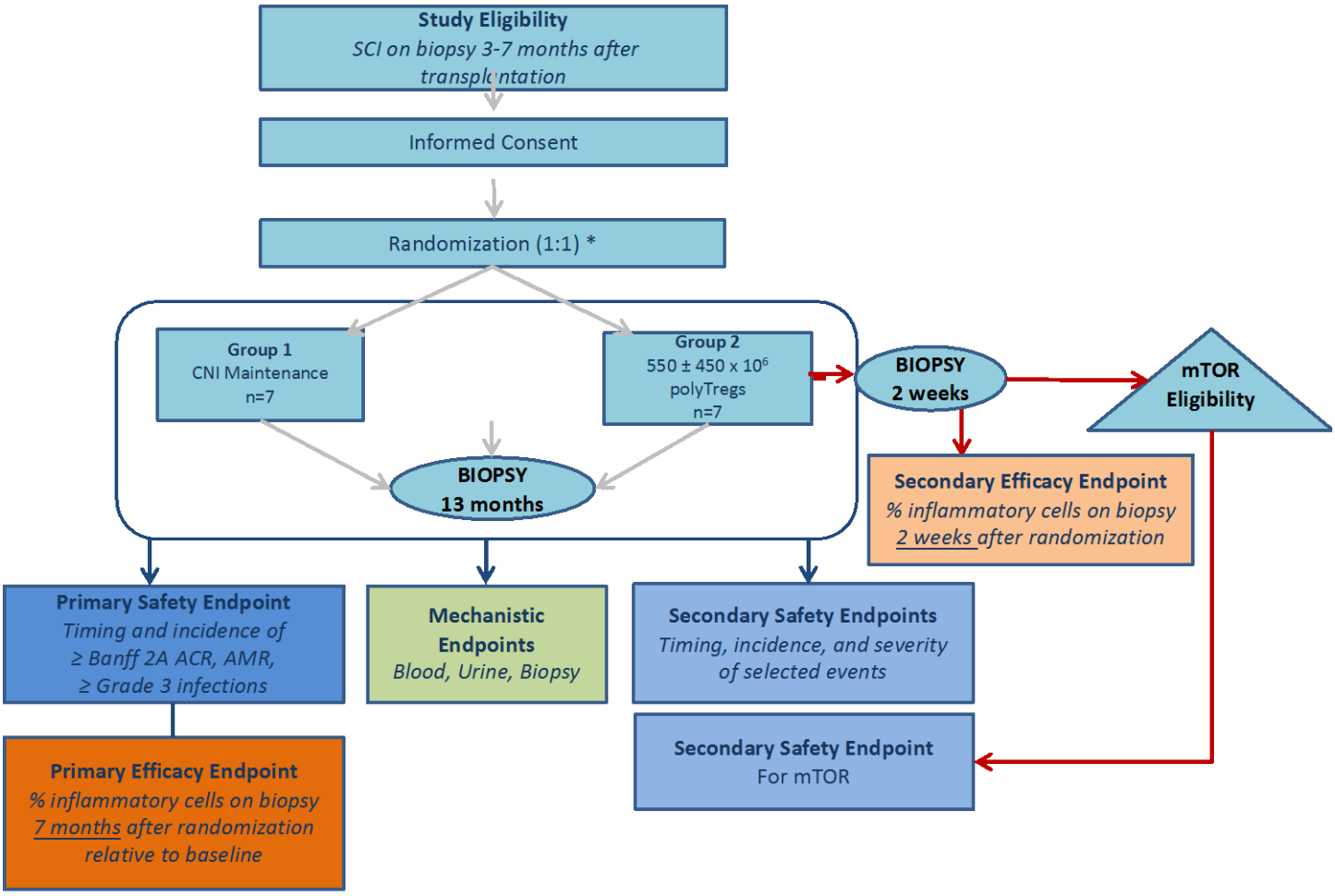
Further, it has been shown that conversion from a CNI to an mTOR inhibitor in transplant recipients is associated with an increase in Treg number and activity (Carroll RP, 2013) (Gallon L T. O.-R., 2015). Animal studies of tolerance induction using Treg infusions have used shown prolonged survival of infused Tregs (Singh K, 2014)and graft acceptance with concomitant sirolimus therapy (Ma A, 2011) (Ma A Q. S., 2009). Therefore, as an added strategy to improve the survival of the infused Tregs, we propose to convert the maintenance immunosuppression from a CNI-based regimen to an everolimus-based regimen in eligible subjects who have safely received polyTregs infusions and have demonstrated a reduction in inflammation on their follow up biopsies at 2 weeks. The previous experiences at UCSF in patients with type 1 diabetes mellitus as well as in a pilot study involving kidney transplant recipients found that infused Tregs reach a peak in the circulation at 2 weeks post-infusion and are still detectable in the circulation at 6 months and 1 year. In animal models, trafficking of infused Tregs in inflamed tissue and draining lymph nodes can be seen within a few hours after infusion. The reduction of inflammatory cell load can be detected in three days, but is typically more pronounced 1 to 2 weeks after the Treg infusion (Lee K, 2014) (Mahne AE, 2015). This delay is likely because Tregs need to accumulate, become activated, and proliferate locally before exerting their suppressive effects. In the clinical setting when we cannot frequently sample the graft, we think it is most likely that we will observe an improvement in graft inflammation at 2 weeks and this will be our signal to initiate a conversion from a CNI to everolimus. Previous studies in kidney transplant recipients have adequately demonstrated the safety of conversion from CNI to everolimus at > 6 months post-transplant (Budde K & Investigators., 2012) (Chhabra D, 2013).

3. Study Design

3.1. Description of Study Design

This is an open-label trial to determine the safety and efficacy of a single dose of autologous polyTregs in renal transplant recipients with SCI in the 3-7 months post-transplant allograft biopsy compared to control patients treated with CNI-based immunosuppression. The efficacy of the Treg therapy will be assessed by the reduction of graft inflammation on biopsies performed at 7 months after study group allocation compared to the eligibility biopsy. The original study design included an additional treatment arm with a single dose of darTregs. However, due to the inability to manufacture an adequate number of cells for infusion, this treatment arm was removed from the study in protocol version 9.0. One subject was treated in the darTreg arm and completed follow-up prior to the arm being removed from the protocol. The accrual goal for the study was reduced due to the removal of this arm as well as challenges associated with recruitment of participants in the setting of the COVID-19 pandemic. Given that this is primarily a pilot, proof-of-concept, we believe that the target of 7 evaluable participants in each arm is sufficient to provide the necessary clinical and mechanistic data that will allow us to assess the impact of polyTregs on graft inflammation.

The study will also describe the safety of mTOR therapy after polyTregs. The secondary efficacy endpoint, as well as safety and mechanistic endpoints relative to both Tregs and mTOR therapy are described below.



*** Initial treatment allocation will weight each treatment equally. However, in subsequent allocations when the groups differ in size by 2 or more, the polyTreg arm will be weighted more in order to restore the balance between the groups.**

Figure 2. Study Design Figure

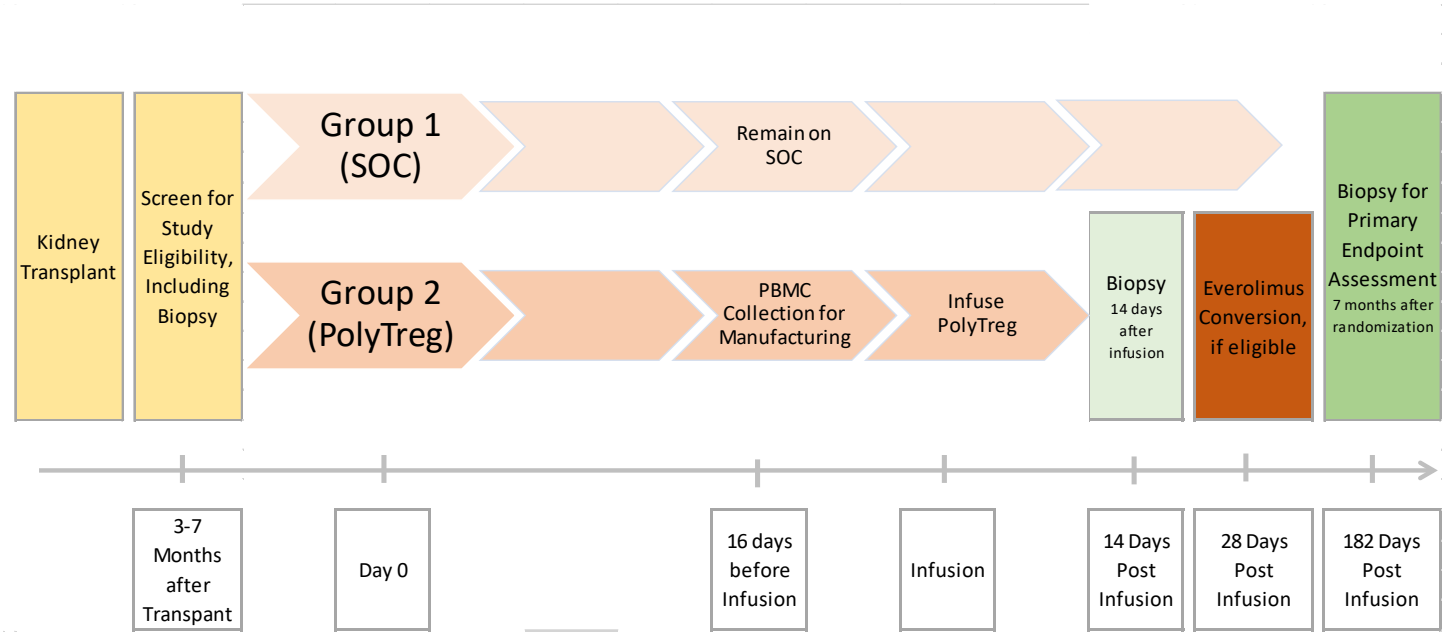


Figure 3. Study Flow/ Timeline Diagram

3.2. Primary Safety Endpoints

The safety of polyTregs (Group 2) will be described in comparison with CNI-based maintenance IS therapy (Group 1) by:

1. The timing and incidence of Banff 2A or higher acute cell-mediated rejection and/or acute antibody mediated rejection
2. The timing and incidence of study defined Grade 3 or higher infection

3.3. Secondary Safety Endpoints

3.3.1. Secondary Safety Endpoints

The safety of PolyTregs (Group 2) will be described in comparison with CNI-based maintenance IS therapy (Group 1) by:

1. Timing, incidence, and severity of polyTregs infusion reaction
2. Timing, incidence, and severity of culture-proven and clinically diagnosed infection after PolyTreg infusion
3. Timing, incidence, and severity of acute rejection using Banff grading
4. Timing and incidence of BK viremia and CMV reactivation
5. Timing and incidence of > 10% decrease in eGFR compared to baseline

3.3.2 Secondary Safety Endpoint for mTOR Therapy

The safety of mTOR therapy after Treg infusion will be assessed by the timing and incidence of acute rejection in Group 2. Subjects who receive Tregs and convert to mTOR therapy will be compared to subjects who did not convert to mTOR therapy; as well as subjects in group 1.

3.4. Primary Efficacy Endpoint

The primary efficacy endpoint will be the change in inflammation as measured by the percentage area of cortex occupied by inflammatory cells using computer-assisted quantitative image analysis on the biopsy 7 months after study group allocation, expressed as the percent change relative to the baseline biopsy.

3.5. Secondary Efficacy Endpoints

The secondary efficacy endpoint will be the proportion of subjects exhibiting a relative decrease of 25% or more inflammation between the baseline kidney biopsy and the biopsy 2 weeks after polyTregs, measured as the percentage area of cortex occupied by inflammatory cells using computer-assisted quantitative image analysis.

Additional secondary efficacy endpoints are the proportion of subjects who exhibit a relative decrease of 50% or more inflammation on kidney biopsy at 2 weeks after polyTregs; and the proportion of subjects who exhibit a relative decrease of 25% or more inflammation at 7 months after study group allocation.

3.6. Primary Mechanistic Endpoints

The primary mechanistic endpoints are the immunologic profiles of kidney transplant recipients using the common response module (CRM) graft gene expression of rejection and/or histologic evidence of inflammation in biopsies at 2 weeks after infusion (Group 2) and 7 months after study group allocation (Groups 1 and 2).

3.6.1. Secondary Mechanistic Endpoints

The secondary mechanistic endpoints are the immunologic profiles of kidney transplant recipients using:

1. Persistence of infused Tregs in blood and biopsies using deuterium labeling and T cell repertoire analysis (Group 2)
2. Cytokine and CRM mRNA and protein profiles in the urine as correlates of acute rejection and/or histologic evidence of inflammation and graft fibrosis (Groups 1 and 2)
3. Peripheral blood (kidney solid organ response test) kSORT mRNA expression of rejection/ increased immune response (Groups 1 and 2)

3.7. Stratification, Study Group Allocation, and Blinding/Masking

This is an open label, parallel group study in which subjects are assigned to treatment alternatives in an unblinded fashion using a variation of the Pocock and Simon adaptive randomization strategy. A web-based randomization system will be used in order to minimize the possibility of bias entering into those assignments and to maintain approximate equality in the group sizes. The initial treatment allocation will weight each treatment equally. In subsequent allocations, the less-populated arm will be weighted more in order to restore the balance between the groups. If a recipient is assigned to polyTreg and the recipient does not receive intended polyTreg infusion, that recipient will be re-assigned to Group 1. The allocation schedule algorithm/scheme will not incorporate stratification variables.

4. Rationale for Study Population

Adult recipients of kidney transplants with stable graft function who are found to have inflammation with or without minor tubulitis on a 6-month surveillance biopsy will be eligible for the trial. Inflammation in the renal allograft is well-recognized as a predictor of long-term graft dysfunction and graft loss ((Park WD, 2010); (Mannon RB M. A., 2010)). Patients who are found to have inflammation on the 6-month surveillance biopsy have already received maximal treatment with induction therapy and are maintained on dual or triple immunosuppression, so more intense therapy may not control the inflammation and is often associated with drug-induced toxicities. Currently, these patients do not receive any specific intervention to treat the inflammation despite the future risk of graft dysfunction. These patients are therefore excellent candidates for immunomodulatory therapy that can alter the balance of the immune system towards a more regulatory phenotype and lead to better long-term function.

4.1. Enrollment Eligibility Criteria

4.1.1. Inclusion Criteria

Individuals who meet all of the following criteria are eligible for enrollment as study participants:

1. Subject must be able to understand and provide informed consent
2. Age ≥18 years of age at the time of study entry
3. Recipients of non-HLA identical living or deceased donor renal transplants
4. Protocol renal allograft biopsy at 5 months (± 8 weeks) after transplantation with Banff i1 and/or ti1 with concomitant t scores t0, t1, t2 or t3; Banff i2 and/or ti2 with concomitant t scores t0 or t1; and without v > 0, [ptc + g] ≥2, C4d >1 (by IF), or C4d > 0 (by IHC); confirmed by central pathologist. Subjects must not be treated for pathologic criteria (e.g. steroids).
5. eGFR ≥30 ml/min at the time of study entry
6. Maintenance immunosuppression consisting of tacrolimus, MMF/MPA ± prednisone (≤10 mg/day)
7. Current immunizations including TdAP, pneumococcal and seasonal influenza vaccines prior to study treatment, completed prior to randomization and no less than 14 days prior to planned manufacturing collection.
8. Hepatitis B serologies must be the following:
    a. Positive HB surface antibody, negative HB core antibody and negative HB surface antigen for recipients immune to hepatitis B
    b. Negative HB surface antibody, negative HB core antibody and negative HB surface antigen for non-immune/ HBV naïve recipients provided donor had negative HB core antibody and negative HB surface antigen at the time of donation
9. Negative TB test (PPD, interferon-gamma release assay, ELISPOT) within 1 year prior to enrollment. Subjects with a history of latent TB (positive TB test without active infection) must have completed one of the current latent TB infection treatment regimens endorsed by the CDC (Division of TB Elimination, 2016).
10. Women of childbearing potential must have reviewed Mycophenolate REMS and have a negative pregnancy test upon study entry
11. Female subjects with child-bearing potential, must agree to use FDA approved methods of birth control for the duration of the study; subjects must consult with their physician and determine the most suitable method(s) that are greater than 80% effective (http://www.fda.gov/birthcontrol)

4.1.2 Exclusion Criteria

Individuals who meet any of these criteria are not eligible for enrollment as study participants:

1. Inability or unwillingness of a participant to give written informed consent or comply with study protocol
2. History of malignancy; except adequately treated basal cell carcinoma
3. History of graft loss from acute rejection within 1 year after any previous transplant
4. History of transplant renal artery stenosis
5. History of cellular rejection prior to enrollment that did not respond to steroids and/or subsequent creatinine after treatment for rejection greater than 15% above baseline
6. Known hypersensitivity to mTOR inhibitors or contraindication to everolimus (including history of wound healing complications)
7. Any chronic illness requiring uninterrupted anti-coagulation after kidney transplantation
8. Post-transplant DSA >5000 MFI or post-transplant treatment with IVIg for DSA. Enrolled subjects with post-transplant DSA >2000 MFI will not be eligible for mTOR conversion.
9. Positive HIV 1 or HIV 2 serology prior to transplantation
10. Known positive HBSAg, or HBcAB serology
11. Proteinuria with urine pr/cr > 1.0 g/g
12. Any condition requiring chronic use of corticosteroids >10mg/day at the time of study entry
13. Subjects requiring treatment for pathologic findings on study eligibility biopsy (see inclusion 4).
14. Active infection at the time of study entry
15. History of active TB or latent TB without adequate treatment (see inclusion 10)
16. Serum BK virus >1,000 copies/ml by PCR at the time of study entry
17. Hematocrit <27%; ANC < 1,000/µL; lymphocytes <500/µL at the time of study entry
18. Participation in any other studies with investigational drugs or regimens in the preceding year
19. Any condition or prior treatment which, in the opinion of the investigator, precludes study participation
20. Unable to provide adequate biopsy specimen (paraffin embedded formalin fixed) from eligibility biopsy (3-7 months post-transplant) for quantitative analysis.
21. EBV naïve recipient of a kidney from an EBV positive donor, historically EBV naïve recipient with primary EBV infection at the time of screening (primary anti-VCA IgM, without antibody to EBNA), positive EBV PCR
22. Hepatitis C Virus Ab positive subjects with negative HCV PCR are eligible if they have spontaneously cleared infection or are in sustained virologic remission for at least 12 weeks after treatment.
23. Positive SARS-CoV2 testing by RT- PCR

4.2. Treg Infusion Eligibility Criteria

4.2.1. Treg Infusion Inclusion Criteria

1. Individuals randomized to group 2 who continue to meet all of the enrollment criteria.
2. Negative SARS-COV2 RTPCR within 1 week of Treg infusion

4.2.2. Treg Infusion Exclusion Criteria

Individuals randomized to group 2 who meet any of these criteria are not eligible for Treg infusion:

1. Received any vaccination within 14 days prior to blood collection for Treg manufacture
2. Unacceptable Treg product
3. Positive pregnancy test for women of childbearing potential

4.3. mTOR Conversion Eligibility Criteria

4.3.1. mTOR Conversion Inclusion Criteria

Individuals who meet all of these criteria are eligible for mTOR conversion:

1. Received at least 300 x 10^6 polyTreg infusion
2. Resolution of inflammation on the 2-week post-infusion biopsy as compared to the baseline biopsy, confirmed by central pathologist

4.3.2 mTOR Conversion Exclusion Criterion

Individuals who meet any of these criteria are not eligible for mTOR conversion:

1. Post-transplant DSA >2000 MFI
2. Any condition or clinical variable, which in the opinion of the site investigator, precludes conversion to mTOR.

4.4. Selection of Clinical Sites

All participating centers use immunosuppressive regimens consisting of tacrolimus, MMF/MPA ± steroids. Based on preliminary data on SCI, 10.9% of those who undergo a protocol kidney biopsy at 6 months will meet histologic criteria for inclusion in the TASK trial. The combined annual volume of both living and deceased kidney transplants at the clinical sites is about 1,927. We are planning to enroll a total of 14 patients, thus requiring a consent rate of approximately 30% which is attainable for the patient population at these clinical centers.

5. Investigational Agent: PolyTregs

5.1. Formulation of PolyTregs

Collection of Recipient T cells

Peripheral blood leukocytes will be collected from eligible participants after eligibility is confirmed. The whole blood or leukapheresed cells will be immediately transported to the manufacturing facility. If the whole blood or leukapheresis product does not produce sufficient numbers of Tregs for infusion, the leukapheresis or phlebotomy may be repeated to ensure an adequate initial cell number for product manufacture.

PolyTregs Expansion

Treg isolated from autologous starting material will be expanded with commercially available anti-CD3 and anti-CD28 magnetic bead for 14 days in cultures containing medium supplemented with deuterated glucose. Blood or leukapheresis product from recipients participating at a clinical site other than UCSF will be shipped at room temperature via next-day service to UCSF for further processing.

5.2. Packaging and Labeling of PolyTregs

At the end of the 14-day expansion of polyTregs, the anti-CD3 and anti-CD28 beads will be removed by magnetic separation. The expanded Tregs will be re-suspended in 100 ml of a solution of 49.02% (v/v) PlasmaLyte-A, 49.02% (v/v) Dextrose 5%, 0.45% NaCl, and 1.96% (v/v) 25% human serum albumin and filled in a sterile infusion bag. The filled product will be refrigerated and quarantined until release.

Prior to release, a product label will be affixed on the infusion bag. The label will provide: the unique product identifier, recommended storage temperature, date of cell harvest, expiration date and time, and clearly indicate the name and unique identifier of the intended recipient. A Certificate of Analysis documenting the test results of release assays will accompany the final product during delivery to the bedside.

The Tregs products manufactured for subjects at an off-site location will be shipped from UCSF to the clinical site via next-day service using validated conditions and containers. Qualified staff at the remote site will receive the polyTregs and verify the recorded shipment temperatures were maintained within the validated range according to UCSF established SOPs.

5.3. Dosage, Preparation, and Administration

Subjects randomized to Group 2 will receive a single infusion of 550 ±450 x 10^6 polyTregs. Preparations less than 100 x 10^6 will not be infused. Cells not infused will be used for research.

Pre-medications will be administered 30-60 minutes prior to infusion. Pre-medications will include 650 mg acetaminophen and 25-50 mg diphenhydramine intravenously or by mouth.

After double verification of cell therapy product and recipient identification, the product will be infused via a peripheral intravenous (IV) line primed with saline by gravity in approximately 20 to 30 minutes. Following administration of the Treg product, product bag, tubing and peripheral IV line will be flushed with normal saline to ensure the complete dose is infused.

Vitals signs will be monitored before, during, and after the infusion. Emergency medical equipment will be available during the infusion in case the subject has an allergic response or an infusion reaction that can result in a CRS. The IV line

will be maintained after the infusion and the subject will be asked to remain in the clinical research unit for a minimum of 24 hours which will allow ongoing monitoring for any infusion-related signs and symptoms.

5.4. Drug Accountability

Under Title 21 of the Code of Federal Regulations (21CFR §312.62) the investigator will maintain adequate records of the disposition of the investigational agent, including the date and quantity of the drug received, to whom the drug was dispensed (participant-by-participant accounting), and a detailed accounting of any drug accidentally or deliberately destroyed.

Records for receipt, storage, use, and disposition will be maintained by the study site. A drug-dispensing log will be kept current for each participant. This log will contain the identification of each participant and the date and quantity of drug dispensed.

All records regarding the disposition of the investigational product will be available for inspection.

5.5. Toxicity Prevention and Management

5.5.1. Dose Escalation Plan

There is no plan for dose escalation in this trial.

5.5.2. Waiting Period between Infusions

Due to manufacturing constraints, there will be a naturally imposed waiting period of at least 1 week in between infusions. This will provide a window to delay or cancel the next infusion in case a stopping rule has been met. Other than the 1-week manufacturing delay, there will be no mandated waiting period between infusions in the polyTregs treatment arm (Group 2), since the proposed starting dose has been infused safely previously in kidney transplant recipients.

5.6. Premature Discontinuation of PolyTregs

PolyTregs will be stopped and will not be restarted if there is a hypersensitivity reaction, a CTCAE ≥ 3 infusion-related reaction, a CTCAE Grade ≥ 3 CRS, or any other infusion related serious adverse event.

6. Investigational Immunosuppressive Regimen: Everolimus

At the time of Treg infusion, the maintenance immunosuppression will consist of tacrolimus with target levels of 4-11 µg/L, MMF/MPA ± steroids. On the follow-up biopsy 2 weeks after the polyTreg infusion, the inflammatory load will be assessed by computer assisted image analysis calculating the number of infiltrating cells per square mm as well as the percentage of renal cortex infiltrated with lymphocytes. If the inflammation has resolved on the 2 week follow up biopsy as compared to the baseline biopsy, everolimus will be started at 1.5 mg bid and the dose of tacrolimus reduced by 50% for 4 weeks and then discontinued. Target everolimus trough levels will be 3-8 µg/L with concomitant tacrolimus therapy, and 6-10 µg/L once tacrolimus is discontinued. These patients will remain on everolimus, MPA ± prednisone through the end of the trial and the follow-up period. If on the 2-week follow up biopsy, inflammation is still present (even if reduced in intensity compared to the baseline biopsy), no change will be made to the maintenance immunosuppressive regimen.

6.1. Zortress® (Everolimus)

Zortress® is an mTOR inhibitor immunosuppressant manufactured by Novartis Pharmaceuticals Corporation and is commercially available. Zortress® is FDA licensed for the prophylaxis of kidney transplant rejection in adults with low to moderate immunologic risk.

6.1.1. Formulation, Packaging, and Labeling

Everolimus is a macrolide immunosuppressant. Everolimus inhibits antigenic and interleukin (IL-2 and IIL-15) stimulated activation and proliferation of T and B lymphocytes. Everolimus is supplied as tablets for oral administration containing 0.25 mg, 0.5 mg, and 0.75 mg of everolimus together with butylated hydroxytoluene, magnesium sterate, lactose monohydrate, hypromellose, crospovidone, and lactose anhydrous as inactive ingredients.

Zortress® tablets are packed in child-resistant blisters. Each strength is available in boxes of 60 tablets (6 blister strips of 10 tablets each). Tablets are white to yellowish, marbled, round, and flat with beveled edges. Tablets should be stored at 25°C (77°F) with excursions permitted to 15-30°C (50-86°F). Tablets should be protected from light and moisture.

6.1.2. Zortress® Prescribing Information (Per Package Insert)

According to the package insert, Zortress® is to be used in combination with basilixamab induction and concurrently with reduced doses of cyclosporine and corticosteroids. An initial everolimus dose of 0.75 mg orally twice daily (1.5 mg/day) is recommended for adult kidney transplant patients in combination with reduced dose cyclosporine, administered as soon as possible after transplantation. Pharmacokinetic studies in kidney transplant patients show that steady-state of everolimus is reached by Day 4. The recommended therapeutic range for everolimus is 3 to 8 ng/mL. Recommended cyclosporine blood concentrations are recommended as follows: 100-200 ng/mL through 1 month after transplant, 75-150 ng/mL at 2 and 3 months after transplant, 50-100 ng/mL at 4 months after transplant, and 25-50 ng/mL from 6 months to 12 months after transplant. Dose adjustments based on everolimus blood concentrations can be made at 4-5 day intervals.

6.1.3. Everolimus Study Dosing and Administration

The use of everolimus in this trial will deviate from package instructions. Subjects in this trial are enrolled between 3 and 7 months after transplantation and induction therapy is site-specific (not study mandated). Furthermore, subjects will be on tacrolimus at study entry, rather than cyclosporine. In eligible patients, everolimus will be initiated within 2 weeks after kidney biopsy showing resolution of inflammation following Treg infusion. Everolimus will be initiated at a dose of 1.5 mg orally twice daily and titrated, as needed. Subjects will begin everolimus with target trough levels of 3-8

µg/L for 4 weeks while still taking tacrolimus. Everolimus target trough levels will be 6-10 µg/L when tacrolimus is discontinued.

6.2. Assessment of Participant Compliance with mTOR and CNI Dosing

Study subjects will have, at a minimum, weekly monitoring of trough levels.

6.3. Toxicity Prevention and Management

In subjects who develop any of the following conditions after initiation of everolimus therapy, everolimus will be discontinued and tacrolimus will be restarted. Target tacrolimus trough levels will be 4-11 mcg/L.

1. Worsening of proteinuria with urine pr/cr >1 g/g
2. Severe hypercholesterolemia (LDL>190 mg/dL or triglycerides >500 mg/dL) not responsive to therapy with lipid lowering agents
3. Severe edema or fluid accumulations such as pleural or pericardial effusions
4. Interstitial pneumonitis
5. Severe mouth ulcers
6. Severe cytopenia (CTCAE grade 3 or higher) not responsive to dose adjustment of everolimus or MMF/ MPA
7. Any condition which in the determination of the investigator requires discontinuation of everolimus therapy such as planned surgery.

6.4. Rejection after Initiation of Everolimus

Any subject with biopsy proven or treated rejection after the start of study mandated everolimus will discontinue everolimus and resume tacrolimus. Target tacrolimus trough levels will be 4-11 mcg/L.

7. Other Medications

7.1. Immunosuppressive Medications

7.1.1. Tacrolimus

Target tacrolimus trough levels for this study prior to conversion to everolimus are 4-11 µg/dl, which falls within standard of care. For eligible subjects in Group 2, the tacrolimus dose will be reduced by 50% when everolimus is initiated. Tacrolimus will be discontinued 4 weeks after initiation of everolimus therapy.

7.1.2 Mycophenolate Mofetil/ Mycophenolic Acid

All enrolled subjects will be on MMF/MPA at the time of study entry at a minimum dose of 1000 mg/720 mg per day. Once subjects are enrolled in the study, doses may be adjusted at the discretion of the study investigator for gastrointestinal intolerance, cytopenias, infections or other conditions that require dose adjustment. All mycophenolate prescribers in the study will be required to enroll in the FDA mycophenolate REMS (risk evaluation and mitigation strategy) program.

7.1.3 Prednisone

Use of corticosteroids is not prohibited in this trial. However, subjects requiring use of corticosteroids >10 mg per day for treatment of long-term conditions (e.g., lupus) will not be enrolled.

7.1.4 Other Immunosuppressive Medications

Subjects requiring the use of cyclosporine and/or azathioprine will not be enrolled in this study.

7.2. Anti-Infective Prophylactic Medications

Anti-infective prophylaxis medications will be per standard of care at each clinical center.

7.3. Prohibited Medications

7.3.1. Non-Leukoreduced Blood Products

For subjects requiring treatment, leukoreduced blood products should be used whenever possible.

7.3.2. Vaccinations

The use of live vaccines will be proscribed during trial participation, as per standard of care for kidney transplant recipients. Examples include (but are not limited to) the following: intranasal influenza, measles, mumps, rubella, oral polio, BCG, yellow fever, varicella, and TY21a typhoid vaccines.

Subjects should not receive any vaccination (live or inactivated) within 14 days prior to blood collection for Treg manufacturing and 28 days after the date of Treg infusion. These requirements are in place to minimize the chance of having enrichment of vaccine specific Tregs in the product and ensure that Treg infusion does not negatively impact immunity elicited by the vaccine.

7.3.3. Medication Interactions

Grapefruit and grapefruit juice inhibit cytochrome P450 3A4 and P-gp activity and should therefore be avoided with concomitant use of EVR. Patients with rare hereditary problems of galactose intolerance, the Lapp lactase deficiency or glucose-galactose mal absorption should not take EVR as this may result in diarrhea and malabsorption.

Administration of medications known to interact with tacrolimus and/or EVR is allowed but tacrolimus and EVR levels should be carefully monitored and dosing titrated to maintain the target levels to minimize toxicity while maintaining efficacy.

7.4. Treatment of Rejection

Patients who are clinically suspected to have a rejection will undergo a kidney biopsy. Biopsies will be read locally according to the Banff criteria and if acute rejection is diagnosed, it will be treated according to the current standard of care. Samples of blood, urine and kidney tissue will be obtained for mechanistic analysis prior to the treatment. Digital images of the biopsies will also be reviewed later by the central pathology core.

8. Study Mandated Procedures

8.1. Blood Draws

Blood draws are necessary after kidney transplantation to monitor allograft function. Whenever possible, study participants will have additional blood drawn for research purposes at time points when standard of care testing is done. Some blood tests will be performed at the local hospital laboratory. Blood for mechanistic assays described in this protocol will also be collected and shipped to core laboratories.

A blood collection of 450-500 mls will be drawn for Treg isolation and manufacture for subjects with hematocrit 30% or higher once the subject is determined to be eligible and randomized to Group 2. Blood collection for this purpose can be repeated once, if necessary, for manufacture of the Treg product (see 8.2 Leukapheresis).

8.2. Leukapheresis

Subjects with hematocrit less than 30% will have leukapheresis performed for PBMC collection. Subjects with hematocrit greater than 30% may undergo leukapheresis or have whole blood collection. The study team will make a decision in the best interest of a particular subject based on the current clinical picture.

8.3. Kidney Biopsy

Study participants will undergo surveillance biopsies as standard of care between 3 and 7 months after transplantation and approximately one year after transplantation. Research specimens will be collected during these procedures and digital images will be sent to the central pathologist for scoring. The biopsy obtained 3-7 months after transplantation will be used to determine study eligibility and local read will be confirmed by central pathologist. The one-year biopsy will fall 7 months after study group assignment and will be used to assess the efficacy endpoint. The one-year biopsy and all for-cause biopsies will be reviewed by the central pathologist.

Additionally, a protocol kidney biopsy will be performed 2 weeks after polyTreg infusion (Group 2). This biopsy is for research purposes only and will be used for mechanistic assays and to confirm eligibility for mTOR conversion. This biopsy will be reviewed by the central pathologist.

9. Known and Potential Risks and Benefits to Participants

9.1. Risks of PolyTregs

Although Treg infusion has been innocuous in animal models, there is currently scant experience in humans. Three Treg therapy trials in GvHD have been reported. The first-in-man trial by Trzonkowski et al involved two patients (Trzonkowski, 2009). The first patient had chronic GvHD two years after transplantation. After receiving 0.1 x 10^6/Kg flow sorted ex vivo expanded Tregs from the donor, the symptoms subsided, and the patient was successfully withdrawn from IS. The second patient had acute GvHD disease that progressed despite three infusions with an accumulative dose of 3 x 10^6/Kg expanded donor Tregs. A larger scale phase I trial led by Brunstein et al (Brunstein, 2010) enrolled twenty- three patients with advanced hematologic malignancy. The patients were treated with two units of umbilical cord blood as source of stem cells and effector T cells. Tregs were isolated using anti-CD25 immunomagnetic bead selection from third-party cord blood samples that had 4 to 6 HLA match with the recipient. Up to 6 x 106/Kg Tregs were infused after ex vivo expansion using anti-CD3 and anti-CD28 conjugated beads. The infused Tregs were detectible in circulation for up to 7 days. During the one-year period after Treg infusion, the investigators observed no DLTs and AEs when compared to historical controls. Incidences of acute and chronic GvHD were reduced in patients received Treg therapy. The third trial enrolled 28 patients with high-risk hematological malignancies (Di Ianni, 2011). Patients received anti-CD25 immunomagnetic bead enriched donor Tregs without ex vivo expansion four days before receiving one haplo-mismatched hematopoietic stem cell and Tconv transplants from the same donors. A majority of the patients received 2 x 106/Kg Tregs with 1 x 106/Kg Tconvs and no adjunct IS was given after transplant. Patients demonstrated accelerated immune reconstitution, reduced CMV reactivation, low incidence of tumor relapse and GvHD. Collectively, these studies show that Treg therapy has minimal toxicity in the setting of GvHD.

Recently, a phase I/II study applying polyclonally expanded FACS purified Tregs to type diabetic patients has been reported by the Trzonkowski group (Marek-Trzonkowska N, 2012). The study enrolled 10 type 1 diabetic children (aged 8-16 years) within 2 months after diagnosis. Four patients received 10 × 10^6 Tregs/kg body wt and the remaining 6 patients received 20 × 10^6 Tregs/kg body weight. The patients were followed for 4-5 months after Treg infusion, and no toxicity of the therapy was noted. The authors concluded that Treg therapy was safe and well tolerated in children.

A phase I Treg dose-escalating trial evaluating safety of Treg therapy in type 1 diabetic patients completed enrollment and infusion in October 2013. Fourteen enrolled subjects received a single infusion of Tregs. Three subjects were treated in each of the first and second dosing cohorts with 0.05x10^8 and 0.4x10^8 cells, respectively, and four subjects were treated in the third dosing cohort with 3.2x10^8 cells. Notably, three of the subjects in cohort 3 and 4 were enrolled at treated at Yale (New Haven, Connecticut). As of August 2014, 115 adverse events had been reported in 15 subjects since the beginning of the trial. All 14 treated subjects have reported at least 1 adverse event. One subject who underwent phlebotomy but was withdrawn before treated reported 1 adverse event before withdrawal from the trial. Seventy-one events were judged as mild in severity, 35 were judged as moderate, and nine were judged as severe. Four events were judged to be serious. Thirty events were judged to be possibly related, 21 unlikely related and 64 unrelated to study therapy. There were no grade 4 or 5 adverse events. Four serious adverse events (SAE) have been reported since the beginning of the trial. Three severe (grade 3) hypoglycemic SAEs, one judged unlikely related and two judged unrelated to the investigational product have been reported. One severe (grade 3) hyperglycemic SAE judged unrelated to the investigational product has been reported.

9.1.1. Potential Risks of Treg Administration

T cell suppression

Tregs are known to suppress naïve T cell responses to a variety of antigens. Less is known about ongoing immune responses especially to viruses and bacteria. It is not known whether Tregs will alter protective immunity. Only recipients with current immunizations including TdAP, pneumococcal and seasonal influenza vaccines will be enrolled in the study. During the flu season, seasonal influenza vaccine will be administered prior to the qualifying biopsy for all transplant recipients and at least 28 days prior to Treg isolation. Subjects immune to hepatitis B can be enrolled in the study; or if non-immune/ HBV naïve, can be enrolled only if their kidney donor has negative HB core antibody and negative HB surface antigen.

Infusion reaction

Side effects reported from previous human trials involving T cell infusions include transient fever, chills, and/or nausea. Subjects will be pre-medicated with acetaminophen 650 mg by mouth and diphenhydramine hydrochloride 25-50 mg by mouth or IV, prior to the infusion of Tregs. These medications may be repeated every six hours as needed. Patients will not receive systemic corticosteroids such as hydrocortisone, prednisone, prednisolone (Solu-Medrol) or dexamethasone (Decadron) at any time, except in the case of a life-threatening emergency, since this may have an adverse effect on T cells. If corticosteroids are required for an acute infusion reaction, an initial dose of hydrocortisone 100 mg may be used.

Infection

As with any therapy that suppresses the immune system, there is a risk of developing infections. It should be noted that on a theoretical basis, this risk is minimal, since the total input of Tregs is approximately 3-5% of the endogenous resident Treg population. We will perform plasma CMV DNA quantitative PCR in all recipients prior to enrollment and exclude those who are positive. Study subjects will be monitored for CMV reactivation by plasma CMV DNA quantitative PCR.

Lymphoproliferative disease

Treg immunosuppression has been shown to enhance tumor growth in some small animal model systems. Thus, complications such as lymphoproliferative disease are possible on a theoretical basis. Clinical experience in transplant recipients suggests that the risk of lymphoproliferative disease is highest in those who develop a primary EBV infection while immunosuppressed. Thus, individuals without prior exposure to EBV are excluded from this study unless the kidney donor is EBV naive. In previously exposed individuals, EBV reactivation is associated with a degree of immunosuppression higher than that likely to be observed in this study since the total input of Tregs is approximately 3-5% of the endogenous resident Treg population. Furthermore, the previously observed rate of PTLD in kidney recipients is relatively low at 0.68% (Smith JM, 2006). Nonetheless, careful attention will be paid to the potential for this complication and we will perform EBV quantitative DNA PCR to screen patients prior to enrollment and monitor for reactivation during the study.

Loss of tumor surveillance

T lymphocytes are one major component of tumor surveillance, and it is possible that cells that inhibit T lymphocytes could impair this function. There has not been evidence of tumors in preclinical models. Nonetheless, the long term follow up of all treated patients will determine whether there is evidence of an increase in the frequency of tumors.

Reproductive risks

There may be an unexpected risk to an unborn or nursing child. Pregnant and breastfeeding women will be excluded from participation in the study. Females must have a negative pregnancy test prior to enrolling in the study. Female participants must agree to not participate in a conception process (e.g., active attempt to become pregnant or in vitro

fertilization) for up to one year after Treg dosing. Females must agree to use a reliable and effective form of birth control for two years after Treg dosing. This includes oral contraceptives, barrier methods, or abstinence. Female subjects will also be required to undergo a urine pregnancy test prior to cell administration. A positive pregnancy test will result in holding of scheduled cell administration.

9.2. Risks of Zortress® (Everolimus)

Boxed warnings for everolimus include increased susceptibility to malignancies and serious infections from immunosuppression; increased risk of kidney arterial and venous thrombosis resulting in graft loss within 30 days post-transplantation; increased nephrotoxicity if used with standard doses of cyclosporine; and increased mortality (associated with serious infections) within the first three months of heart transplantation.

In this study, the period of overlap between everolimus and a CNI (tacrolimus) will be 4 weeks; and during this period tacrolimus will be administered at a reduced dose. The risks that have been noted previously with combination therapy of cyclosporine and everolimus are therefore not anticipated in this trial. Risks applicable to the use of everolimus in this study include:

Angioedema

Everolimus has been associated with the development of angioedema. The concomitant use of everolimus with other drugs known to cause angioedema, such as angiotensin converting enzyme (ACE) inhibitors may increase the risk.

Wound Healing and Fluid Accumulation

Everolimus delays wound healing and increases the occurrence of wound-related complications like wound dehiscence, wound infection, incisional hernia, lymphocele and seroma. These wound-related complications may require more surgical intervention. Generalized fluid accumulation, including peripheral edema (e.g., lymphoedema) and other types of localized fluid collection, such as pericardial and pleural effusions and ascites have also been reported.

Hyperlipidemia

Increased serum cholesterol and triglycerides, requiring the need for anti-lipid therapy, have been reported to occur following initiation of everolimus and the risk of hyperlipidemia is increased with higher everolimus whole blood trough concentrations. Use of anti-lipid therapy may not normalize lipid levels in patients receiving everolimus.

Due to an interaction with cyclosporine, clinical trials of everolimus and cyclosporine in kidney transplant patients strongly discouraged patients from receiving the HMG-CoA reductase inhibitors simvastatin and lovastatin. During everolimus therapy with cyclosporine, patients administered an HMG-CoA reductase inhibitor and/or fibrate should be monitored for the possible development of rhabdomyolysis and other adverse effects, as described in the respective labeling for these agents.

Proteinuria

The use of everolimus with cyclosporine in transplant patients has been associated with increased proteinuria. The risk of proteinuria increased with higher everolimus whole blood trough concentrations.

Polyoma Virus Infections

Patients receiving immunosuppressants, including everolimus, are at increased risk for opportunistic infections; including polyoma virus infections. Polyoma virus infections in transplant patients may have serious, and sometimes fatal, outcomes. These include polyoma virus-associated nephropathy (PVAN), mostly due to BK virus infection, and JC virus associated progressive multiple leukoencephalopathy (PML). PVAN has been observed in patients receiving immunosuppressants, including everolimus.

Non-Infectious Pneumonitis

A diagnosis of non-infectious pneumonitis should be considered in patients presenting with symptoms consistent with infectious pneumonia or radiologic changes in whom infectious, neoplastic and other non-drug causes have been ruled out through appropriate investigations. Fatal cases have been reported. Non-infectious pneumonitis may respond to drug interruption with or without glucocorticoid therapy.

Thrombotic Microangiopathy/ Thrombotic Thrombocytopenic Purpura/ Hemolytic Uremic Syndrome (TMA/TTP/HUS)

The concomitant use of everolimus with cyclosporine may increase the risk of thrombotic microangiopathy/thrombotic thrombocytopenic purpura/hemolytic uremic syndrome.

New Onset Diabetes after Transplant

Everolimus has been shown to increase the risk of new onset diabetes mellitus after transplant.

Male Infertility

Azospermia or oligospermia may be observed. Everolimus affects rapidly dividing cells like the germ cells which can lead to reduced male fertility. These effects are reversible with discontinuation of therapy.

Pregnancy and Nursing

Everolimus is a pregnancy category C drug. In animal studies, everolimus crossed the placenta and was toxic to the conceptus. The potential risk for humans is unknown. Women of childbearing potential should be advised to use effective contraception methods while they are receiving everolimus and up to 8 weeks after treatment has been stopped. Because many drugs are excreted in human milk and because of the potential for serious adverse reactions in nursing infants from everolimus, women should avoid breast-feeding during treatment with everolimus.

9.3. Risks of Tacrolimus and Mycophenolate Mofetil/Mycophenolic Acid

Subjects will be on CNI and mycophenolate at the time of study entry. Potential risks applicable to their use in this study are listed below.

9.3.1. Risks of Tacrolimus

Post-Transplant Diabetes Mellitus

Insulin-dependent post-transplant diabetes mellitus (PTDM) was reported in 20% of Prograf-treated kidney transplant patients without pretransplant history of diabetes mellitus in the Phase III study. The median time to onset of PTDM was 68 days. Insulin dependence was reversible in 15% of these PTDM patients at one year and in 50% at 2 years post-transplant. Black and Hispanic kidney transplant patients were at an increased risk of development of PTDM.

Nephrotoxicity

Tacrolimus can cause nephrotoxicity, particularly when used in high doses. Nephrotoxicity was reported in approximately 52% of kidney transplantation patients and in 40% and 36% of liver transplantation patients receiving tacrolimus in the U.S. and European randomized trials, respectively, and in 59% of heart transplantation patients in a European randomized trial. In patients with persistent elevations of serum creatinine who are unresponsive to dosage adjustments, consideration should be given to changing to another immunosuppressive therapy. Care should be taken in using tacrolimus with other nephrotoxic drugs.

Hyperkalemia

Mild to severe hyperkalemia was reported in 31% of kidney transplant recipients and in 45% and 13% of liver transplant recipients treated with tacrolimus in the U.S. and European randomized trials, respectively, and in 8% of heart transplant

recipients in a European randomized trial and may require treatment. Serum potassium levels should be monitored and potassium-sparing diuretics should not be used during tacrolimus therapy.

Neurotoxicity

Tacrolimus can cause neurotoxicity, particularly when used in high doses. Neurotoxicity, including tremor, headache, and other changes in motor function, mental status, and sensory function were reported in approximately 55% of liver transplant recipients in the two randomized studies. Tremor occurred more often in tacrolimus-treated kidney transplant patients (54%) and heart transplant patients (15%) compared to cyclosporine-treated patients. The incidence of other neurological events in kidney transplant and heart transplant patients was similar in the two treatment groups. Tremor and headache have been associated with high whole-blood concentrations of tacrolimus and may respond to dosage adjustment. Seizures have occurred in adult and pediatric patients receiving tacrolimus. Coma and delirium also have been associated with high plasma concentrations of tacrolimus. Patients treated with tacrolimus have been reported to develop posterior reversible encephalopathy syndrome (PRES). Symptoms indicating PRES include headache, altered mental status, seizures, visual disturbances and hypertension. Diagnosis may be confirmed by radiological procedure. If PRES is suspected or diagnosed, blood pressure control should be maintained and immediate reduction of immunosuppression is advised. This syndrome is characterized by reversal of symptoms upon reduction or discontinuation of immunosuppression.

Malignancy and Lymphoproliferative Disorders

As in patients receiving other immunosuppressants, patients receiving tacrolimus are at increased risk of developing lymphomas and other malignancies, particularly of the skin. The risk appears to be related to the intensity and duration of immunosuppression rather than to the use of any specific agent. A lymphoproliferative disorder (LPD) related to Epstein-Barr Virus (EBV) infection has been reported in immunosuppressed organ transplant recipients. The risk of LPD appears greatest in young children who are at risk for primary EBV infection while immunosuppressed or who are switched to tacrolimus following long-term immunosuppression therapy. Because of the danger of over suppression of the immune system which can increase susceptibility to infection, combination immunosuppressant therapy should be used with caution.

Latent Viral Infections

Immunosuppressed patients are at increased risk for opportunistic infections, including activation of latent viral infections. These include BK virus associated nephropathy and JC virus associated progressive multifocal leukoencephalopathy (PML) which have been observed in patients receiving tacrolimus. These infections may lead to serious, including fatal, outcomes.

Tacrolimus in Combination with Sirolimus

The use of full-dose tacrolimus with sirolimus (2 mg per day) in heart transplant recipients was associated with increased risk of wound healing complications, renal function impairment, and insulin-dependent post-transplant diabetes mellitus, and is not recommended. Study subjects will not be prescribed full-dose tacrolimus in this trial. Rather, subjects will be weaned off tacrolimus once target everolimus levels have been reached.

9.3.2. Risks of Mycophenolate Mofetil/ Mycophenolic Acid

Embryofetal Toxicity

Mycophenolate mofetil (MMF) can cause fetal harm when administered to a pregnant female. Use of MMF during pregnancy is associated with an increased risk of first trimester pregnancy loss and an increased risk of congenital malformations, especially external ear and other facial abnormalities including cleft lip and palate, and anomalies of the distal limbs, heart, esophagus, kidney and nervous system.

All prescribers participating in the study will be required to enroll in the FDA mycophenolate REMS program. Of note, all study participants will already have been on a maintenance regimen containing MMF/MPA and will not be started on it as part of the study. All females must be willing to use FDA approved methods of birth control acceptable during the entire period of the study. Urine pregnancy test is part of the screening laboratory tests and will be repeated prior to polyTreg infusion.

For those females who are discovered to be pregnant either at study screening or enrollment or during the study and who are on MMF/MPA or within 6 weeks of discontinuing therapy, the study investigators will report the pregnancy to the Mycophenolate Pregnancy registry (1-800-617-8191) and strongly encourage the patient to enroll in the pregnancy registry. When appropriate, pregnant patients will be switched to alternative immunosuppression with less potential for embryo-fetal toxicity after a discussion of maternal and fetal risks and benefits.

Lymphoma and Malignancy

Patients receiving immunosuppressive regimens involving combinations of drugs, including MMF, as part of an immunosuppressive regimen are at increased risk of developing lymphomas and other malignancies, particularly of the skin. The risk appears to be related to the intensity and duration of immunosuppression rather than to the use of any specific agent. As usual for patients with increased risk for skin cancer, exposure to sunlight and UV light should be limited by wearing protective clothing and using a sunscreen with a high protection factor. Lymphoproliferative disease or lymphoma developed in 0.4% to 1% of patients receiving MMF (2 g or 3 g) with other immunosuppressive agents in controlled clinical trials of renal, cardiac, and hepatic transplant patients. In pediatric patients, no other malignancies besides lymphoproliferative disorder (2/148 patients) have been observed.

Combination with Other Immunosuppressive Agents

MMF has been administered in combination with the following agents in clinical trials: antithymocyte globulin (ATGAM®), OKT3 (Orthoclone OKT® 3), cyclosporine (Sandimmune®, Neoral®) and corticosteroids. The efficacy and safety of the use of MMF in combination with other immunosuppressive agents have not been determined.

Serious Infections

Patients receiving immunosuppressants, including MMF, are at increased risk of developing bacterial, fungal, protozoal and new or reactivated viral infections, including opportunistic infections. These infections may lead to serious, including fatal outcomes. Because of the danger of over suppression of the immune system which can increase susceptibility to infection, combination immunosuppressant therapy should be used with caution.

New or Reactivated Viral Infections

Polyomavirus associated nephropathy (PVAN), JC virus associated progressive multifocal leukoencephalopathy (PML), cytomegalovirus (CMV) infections, reactivation of hepatitis B (HBV) or hepatitis C (HCV) have been reported in patients treated with immunosuppressants, including MMF. Reduction in immunosuppression should be considered for patients who develop evidence of new or reactivated viral infections. Physicians should also consider the risk that reduced immunosuppression represents to the functioning allograft.

PML, which is sometimes fatal, commonly presents with hemiparesis, apathy, confusion, cognitive deficiencies, and ataxia. Risk factors for PML include treatment with immunosuppressant therapies and impairment of immune function. In immunosuppressed patients, physicians should consider PML in the differential diagnosis in patients reporting neurological symptoms and consultation with a neurologist should be considered as clinically indicated. The risk of CMV viremia and CMV disease is highest among transplant recipients seronegative for CMV at time of transplant who receive a graft from a CMV seropositive donor. Therapeutic approaches to limiting CMV disease exist and should be routinely

provided. Patient monitoring may help detect patients at risk for CMV disease. Viral reactivation has been reported in patients infected with HBV or HCV. Monitoring infected patients for clinical and laboratory signs of active HBV or HCV infection is recommended.

Neutropenia

Severe neutropenia [absolute neutrophil count (ANC) <0.5 x 10^3/uL] developed in up to 2.0% of renal, up to 2.8% of cardiac, and up to 3.6% of hepatic transplant patients receiving MMF 3g daily. Patients receiving MMF should be monitored for neutropenia. The development of neutropenia may be related to CellCept itself, concomitant medications, viral infections, or some combination of these causes. If neutropenia develops (ANC <1.3 x 10^3/uL), dosing with MMF should be interrupted or the dose reduced, appropriate diagnostic tests performed, and the patient managed appropriately. Neutropenia has been observed most frequently in the period from 31 to 180 days post-transplant in patients treated for prevention of renal, cardiac, and hepatic rejection.

Patients receiving MMF should be instructed to report immediately any evidence of infection, unexpected bruising, bleeding, or any other manifestation of bone marrow depression.

Pure Red Cell Aplasia (PRCA)

Cases of pure red cell aplasia (PRCA) have been reported in patients treated with MMF in combination with other immunosuppressive agents. The mechanism for MMF induced PRCA is unknown; the relative contribution of other immunosuppressants and their combinations in an immunosuppression regimen are also unknown. In some cases, PRCA was found to be reversible with dose reduction or cessation of MMF therapy. In transplant patients, however, reduced immunosuppression may place the graft at risk.

Gastrointestinal Disorders

Gastrointestinal bleeding (requiring hospitalization) has been observed in approximately 3% of renal, in 1.7% of cardiac, and in 5.4% of hepatic transplant patients treated with MMF 3 g daily. In pediatric renal transplant patients, 5/148 cases of gastrointestinal bleeding (requiring hospitalization) were observed. Gastrointestinal perforations have rarely been observed. Most patients receiving MMF were also receiving other drugs known to be associated with these complications. Patients with active peptic ulcer disease were excluded from enrollment in studies with MMF. Because MMF has been associated with an increased incidence of digestive system adverse events, including infrequent cases of gastrointestinal tract ulceration, hemorrhage, and perforation, MMF should be administered with caution in patients with active serious digestive system disease.

Patients with Renal Impairment

Subjects with severe chronic renal impairment (GFR <25 mL/min/1.73 m^2) who have received single doses of MMF showed higher plasma MPA and MPAG AUCs relative to subjects with lesser degrees of renal impairment or normal healthy volunteers. No data are available on the safety of long-term exposure to these levels of MPAG. Doses of MMF greater than 1 g administered twice a day to renal transplant patients should be avoided and they should be carefully observed.

Patients with HGPRT Deficiency

MMF is an IMPDH (inosine monophosphate dehydrogenase) inhibitor; therefore, it should be avoided in patients with rare hereditary deficiency of hypoxanthine-guanine phosphoribosyl-transferase (HGPRT) such as Lesch-Nyhan and Kelley-Seegmiller syndrome.

Phenylketonurics

MMF Oral Suspension contains aspartame, a source of phenylalanine (0.56 mg phenylalanine/mL suspension). Therefore, care should be taken if MMF Oral Suspension is administered to patients with phenylketonuria.

9.4. Risks of Study Procedures

9.4.1. Risks of Blood Draws

Risks of blood draw or venipuncture are typically minimal with temporary local discomfort. More serious risks would include ecchymosis and, rarely, localized infection. The amount of blood that may be drawn from adult subjects for research purposes will not be more than 600 mL over an eight-week period. The additional amount of blood could contribute to the development of anemia. The subject's clinical condition will be taken into consideration to determine if research blood tests can be performed.

9.4.2. Risks of Leukapheresis

The common risks of leukapheresis include bruising and discomfort at the site of needle placement, typically in the antecubital fossae. Calcium level in blood may fall due the citrate anticoagulant used to prevent clotting in the leukapheresis instrument. Hypocalcemia can lead to perioral or digital numbness and tingling. Calcium replacement may be used during the procedure and is routinely used at the conclusion of the procedure. Platelet count may fall due to platelet loss during processing. Hemorrhagic complications due to thrombocytopenia have not been reported in normal donors. Thrombosis and bleeding could theoretically occur, although they are rarely if ever observed.

9.4.3. Risks of Kidney Biopsy

There is a risk of bleeding associated with transplant kidney biopsies. Transient hematuria occurs in 3 to 10% of patients and may prolong hospitalization, require bladder catheterization for clot drainage, or in approximately 1% of patients, require blood transfusion. Ureteral obstruction from blood clot may require percutaneous nephrostomy in <1% of patients. Massive hemorrhage requiring surgical exploration, transplant nephrectomy, or arterial embolization occurs in ~0.1 % of patients. Death from massive hemorrhage is rare.

9.5. Potential Benefits

This study might not provide direct or immediate benefit to the participants.

9.5.1. Decrease in Graft Inflammation

It is possible that infusions of Tregs may modify the infiltrate in the renal allograft to the benefit of the kidney (Taflin C, 2010).

9.5.2. CNI Reduction

Subjects who have a significant decrease in graft inflammation will be eligible for reduction and potentially elimination of tacrolimus therapy. This may help to reduce/ prevent adverse effects associated with CNI therapy, including nephrotoxicity (Mjörnstedt L, 2012).

10. Study Visits

10.1. Enrollment

The research study will be explained in lay terms to each potential research participant. The potential participant will sign an informed consent form before undergoing any study procedures. A subject is considered enrolled in the trial once the consent form has been signed.

10.2. Study Eligibility and Study Group Allocation

Subjects will have a renal allograft biopsy performed 5 months (±8 weeks) after transplantation. Biopsy results will be an integral part of screening for study eligibility and will be confirmed by the central pathologist prior to enrollment. During the screening period the study personnel will review the subject's medical record for previous and current medical history, demographic information (age, gender, and race), medications, and laboratory test results. Additional laboratory tests, such as a pregnancy test (if applicable), will be performed at the transplant center for screening purposes. Blood, urine and tissue will be collected for central laboratory analysis.

Eligible patients will be assigned according to the allocation scheme (Section 3.7) to 2 groups: group 1 CNI maintenance; group 2 infusion of 550 ± 450 x 10^6 polyTregs. The day of study group assignment is designated day 0 on the Schedule of Events (Appendix 2 and 3).

10.3. Specific Visits for Group 2

10.3.1. Whole Blood Collection or Leukapheresis for Treg Manufacture (PBMC Collection Visit)

Participants in Group 2 will provide 450-500 ml of whole blood via phlebotomy or have leukapheresis; either will be shipped to UCSF for manufacturing. The actual visit dates of recipient collections will be based on the selected infusion date.

10.3.2. Treg Infusion Visit

Eligibility for Treg infusion will be reviewed at the time of the study visit. When applicable, a urine pregnancy test must be confirmed negative prior to infusion. Blood will be drawn for local laboratory tests. Subjects will receive the polyTreg infusion no later than 62 days after central biopsy confirmation. The Treg infusion visit requires an overnight stay in the hospital or research unit for observation.

10.4. Follow Up Visits

Group 2 participants will have study visits on day 1, 7, 14, 28, 84, 182, and 364 after Treg infusion. Participants in Group 1 will have the same visits in parallel with Group 2 patients, starting 48 days after study group assignment. Please refer to Appendix 2 for Group 1 schedule of events and Appendix 3 for Group 2 schedule of events.

10.4.1 Provisions for COVID-19

Participating centers should follow site-specific guidance related to COVID-19 in regard to telemedicine visits in leiu of visits at the study site. For CTOT-21, Visit 2/ Day 14 and Visit 5/ Day 182 are considered essential or ciritical for assessment study endpoints. Efforts should be made to have subjects return to the study site for these two visits in order to obtain required assessments, including blood, biopsy, and urine specimens. In addition, study assessments should be obtained if a subject has a clinically indicated biopsy.

10.5. Unscheduled Visits/ Clinically Indicated Biopsies

If creatinine increases or other concerns arise between regularly scheduled visits, participants will be instructed to return to the study site for an "unscheduled" visit. Study assessments will be performed, and research specimens collected if a subject has a clinically indicated biopsy. Please see Appendix 2 for details.

10.6. Visit Windows

Study visits should take place within the time limits specified below (Table 2). The designated visit windows for each scheduled visit are also indicated on the Schedules of Events.

Table 2. Visit Windows

Study Visit	**Visit Window**
Study Eligibility Biopsy	5 months ± 8 weeks
Study Eligibility/ Study Group Allocation	≤ 2 weeks after qualifying biopsy
Group 1 Only	
Day 41/ Visit T0-G1	± 3 days
Day 48/ Visit 1 Day 55/ Visit 2 Day 69/ Visit 3 Day 125/ Visit 4	± 5 days
Day 223/ Visit 5	± 14 days
Day 405/ Visit 6	
Group 2	
PBMC Collection Visit T-16	Dictated by infusion date and manufacturing timeline.
Treg Infusion Day / Visit T0	Infusion can occur any time up to and including day 62 after central pathology confirmation.
Visit T1 / Day 1	± 1 hour (Group 2)
Visit 1 / Day 7	± 1 day
Visit 2 / Day 14 Visit 3 / Day 28 Visit 4 / Day 84	± 3 days
Visit 5 / Day 182	± 14 days
Visit 6 / Day 364	± 14 days

11. Mechanistic Assays

The goal of the mechanistic studies in this trial is to evaluate the impact of polyTregs on the immunological profiles of kidney transplant recipients. We will accomplish this by determining: 1) impact of Treg infusion on the numbers of circulating and graft-infiltrating Tregs; 2) impact of Treg infusion on the inflammation in the graft; 3) impact of Treg infusion on the peripheral blood biomarkers of rejection and graft injury.

11.1. Impact of Treg Infusion on Numbers of Circulating and Graft-Infiltrating Tregs

11.1.1. Detection of Infused Tregs Using Deuterium Labeling (Deu-bl)

Infused polyTregs will be indistinguishable from endogenous Tregs by standard surface markers, prohibiting the tracking of infused cells using conventional means. The Hellerstein group and others have developed stable-isotope-labeling technology to measure the survival, replication, and trafficking of murine and human cells. Importantly, deuterium, a naturally occurring stable isotope of hydrogen, is nonradioactive and non-toxic, and has been safely used as a cellular, molecular, and metabolic marker in patients and healthy controls for more than 6 decades (Busch R, 2007) (Macallan DC, 1998). The use of deuterium-labeled glucose in polyTreg cultures led to ~60% labeling of DNA that was detectable using gas chromatography-mass spectrometry (GC-MS). Deuterium labeling is permanent such that the percentage of deuterated DNA on a per cell basis only decreases with DNA replication. After intravenous administration of 3.2 x 10^8 deuterium-labeled polyTregs in the UCSF type I diabetes trial, infused Tregs were readily detected up to 180 days post infusion in all 3 patients, demonstrating the feasibility of this approach (Figure 4). In addition, early experience with a kidney transplant recipient shows that Tregs from immunosuppressed patients can be labeled with similar efficiency during in vitro expansion and the in vivo pharmacokinetics of Tregs is similar to that seen type 1 diabetes patients who were not on immunosuppression (Figure 4).

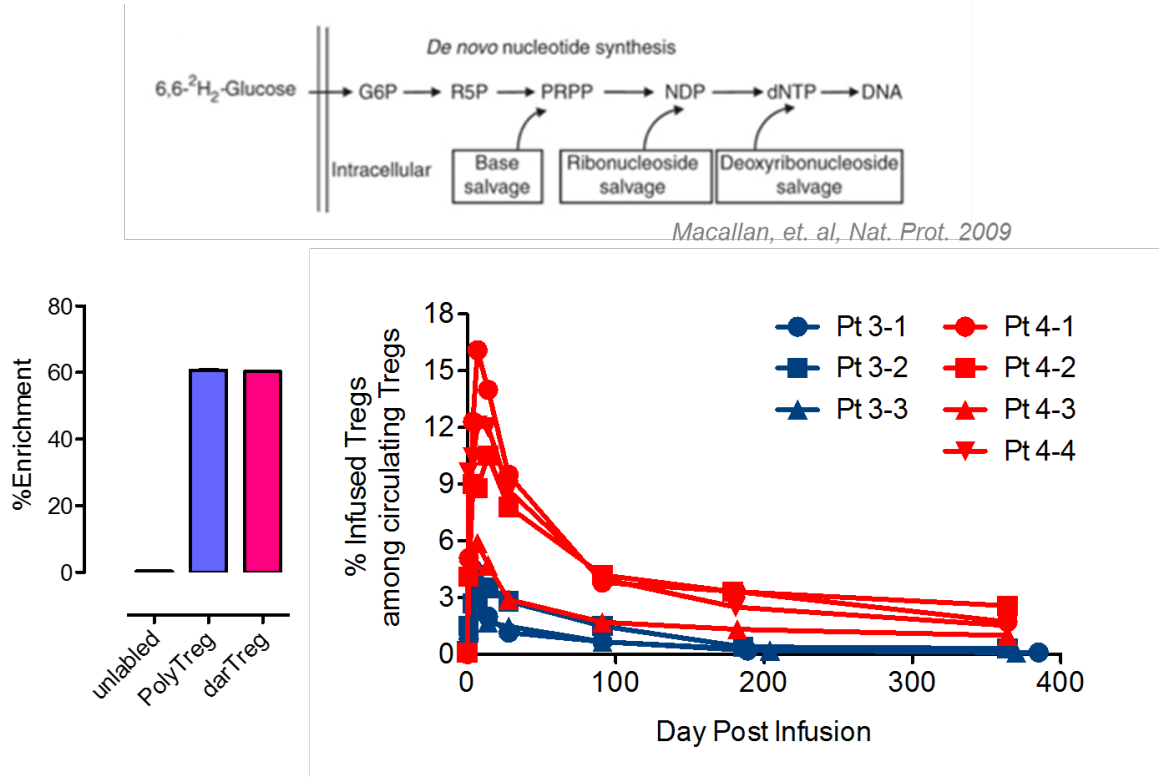


Figure 4. Deuterium labeling for Treg tracking

Following infusion of stable isotope-labeled Tregs, 15 ml of whole blood will be collected at visits specified in the schedule of events (Appendix 2 and 3). The cells will be processed into PBMC and Tregs will be sorted based on $CD4^+CD127^{lo/-}CD25^+$ markers. In addition, $CD4^+$ T cells that are outside the Treg gate will also be collected and analyzed to determine if infused Tregs give rise to non-Tregs in vivo after infusion. Genomic DNA will be isolated from the purified cells and hydrolyzed before assessing the ^2H isotopic enrichment of the purine deoxyribonucleosides using GC-MS. The level of ^2H enrichment among the purified Treg and non-Treg cells will be determined.

11.1.2. Detection of Infused Tregs in Graft Using Deuterium Labeling (Deu-biopsy)

Tregs have a propensity to traffic to and accumulate in sites of inflammation. Since patients in this trial are selected to have inflammation in the kidney allograft, the infused Tregs may infiltrate the graft. To determine if this occurs, fresh biopsy samples (1/2 to a full 16G core) will be collected 2 weeks after Treg infusion. The tissue will be digested to dissociate the cells into single cell suspension. Infiltrating $CD45^+$immune cells and its various subsets will be isolated using FACS and the level of ^2H enrichment among the purified cells will be determined as described above for blood samples.

11.1.3. Peripheral Blood Treg Number (MFC-Treg)

Blood (1 ml) will be collected at visits specified in the schedule of events (Appendix 2 and 3), processed into PBMC, and cryopreserved for batched analysis. Percentage of Tregs among CD4 T cells will be determined using multiparameter flow cytometry (MFC) as described in section 11.3.1 below. The absolute number of peripheral blood Tregs will be calculated using the number of CD4 counts obtained from clinical lab test.

11.1.4. Treg TruCount Analysis

Samples collected for this assay will be used directly for analysis without cryopreservation. Blood will be aliquoted into a TruCount tube, stained with fluorochrome-conjugated antibodies to CD4, CD45, CD25 and CD127, and analyzed on a flow cytometer to enumerate the numbers of Tregs.

This assay would allow us to obtain the absolute Treg counts in one microliter of blood.

11.2. Impact of Treg Infusion on Graft Inflammation

11.2.1. Detection of Intragraft Inflammation by Multiplexed Immunofluorescence and *in situ* Hybridization (mIF/ISH)

Histopathology combined with immunohistochemistry (IHC) or immunofluorescence (IF) provides information on the location, nature, and magnitude of the inflammatory response, but traditionally is not a reliable approach for quantifying changes in inflammatory mediators. To quantitatively assess cytokine expression in the graft tissue in response to intervention, the Laszik lab has adapted the RNAScope® *in situ* hybridization (ISH) platform to analyze formalin-fixed paraffin-embedded biopsies. Furthermore, a novel technology that combines ISH with IF was developed and optimized for various targets; this highly sensitive and specific ISH/IF methodology combined with computer-assisted image analysis allows precise quantitative phenotypical analysis of immune infiltrates and parenchymal cells along with their gene expression profiles in the biopsy tissue. We plan to use this technology to assess change in inflammatory and tolerance markers in the grafts after Treg therapy in biopsy samples obtained at 2 weeks and 6 months after Treg infusion and compared to the baseline in the eligibility biopsy samples. The following structural and inflammatory markers will be evaluated using IHC/IF technology: collagen 3 for interstitial fibrosis, and CD4, CD8, Treg (FoxP3), CD20, and CD68 for inflammation. ISH IL-6 mRNA will be detected via ISH. Any for-cause biopsies performed during the study will also be evaluated using the same parameters.

11.2.2. Graft Common Response Module (CRM) Gene Expression

Increased transcriptional profiling of transplant biopsies has provided useful insights into allograft injury mechanisms such as acute and chronic rejection (Samanta A, 2008) (Morgun A, 2006) (Snyder TM, 2011). Identifying a common rejection mechanism could facilitate novel diagnostics without requiring details about tissue-specific injury. Recently, the Sarwal Lab and collaborators analyzed whole genome expression profiles in 13 independent transplant cohorts consisting of 1164 graft biopsy samples from four different organs (kidney, heart, lung, and liver) and thus representing the largest study of its kind in transplantation. This study was able to define a CRM for the prediction of cross-organ acute rejection and identification of novel drug targets in transplantation (Khatri P, 2013). The CRM consists of 12 genes that were significantly up regulated during rejection independent of the organ type (Figure 5 and Table 3). Additional pathway analyses revealed the relevant association of these genes with T cell receptors, interleukins, chemokines and transcription factors. In order to avoid (1) the influence of a single large experiment on the meta-analysis results and (2) organ-specific bias due to unequal number of data sets (and samples) used in the metaanalysis, a “leave-one-organ out” meta-analysis was performed leading to the definition of 12 ubiquitously overexpressed rejection associated genes BASP1, CD6, CD7, CXCL10, CXCL9, INPP5D, ISG20, LCK, NKG7, PSMB9, RUNX3, and TAP1.

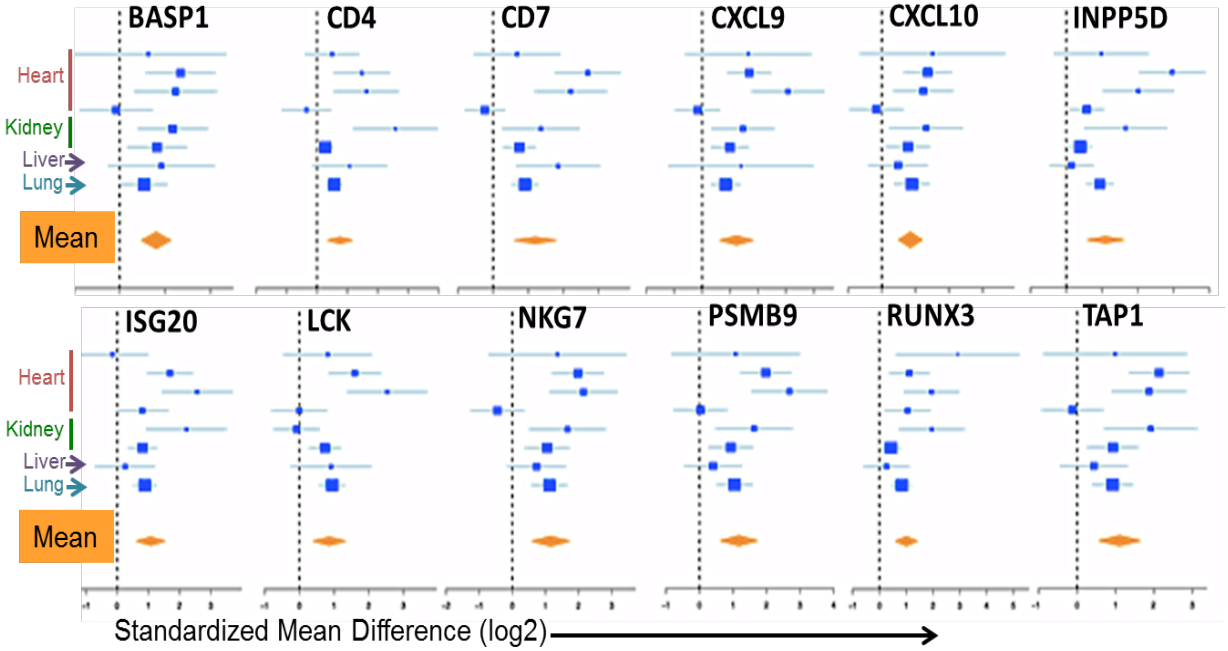


Figure 5. 12-Gene CRM across heterogeneous microarray datasets on different platforms.

Gene ID	Protein name	Function
BASP1	Brain acid soluble protein 1	Transmembrane protein involved in signaling, found in renal epithelial cells and other cells with high turnover
CD6	T-cell differentiation antigen CD6	Surface protein expressed on lymphocytes, involved in cell adhesion
CXCL9	C-X-C motif chemokine 9	Cytokine that affects growth, movement, or activation of immune cells, recruits T-cells.
CXCL10	C-X-C motif chemokine 10	Cytokine that recruits monocytes and T-cells.
INPP5D	Phosphatidylinositol 3,4,5-triphosphatase 1	Negative regulator of B-cell antigen receptor signaling
ISG20	Interferon-stimulated gene 20kDa	Cleaves single-stranded RNA and DNA; induced by interferon gamma (IFNγ) has antiviral effects against RNA viruses
LCK	Tyrosine-protein kinase Lck	Protein kinase involved in signaling to promote T cell response and proliferation
NKG7	Natural killer cell protein 7	Surface membrane protein expressed on activated T cells, kidney, liver and pancreas
PSMB9	Proteasome subunit beta type-9	Cleaves proteins to create MHC class I binding peptides
RUNX3	Runt-related transcription factor 3	Regulates transcription by binding enhancers and promoters of polyomavirus, T-cell receptor, lck, IL-2 and GM-CSF
TAP1	Antigen peptide transporter 1	Transports antigens into endoplasmic reticulum for presentation by MHC class I

Table 3. CRM: Upregulated genes during rejection

The significance of the CRM genes in AR was validated in an independent cohort of publicly available microarray data from 282 renal allograft biopsies (GSE 36059; (Halloran PF, 2010)) and classified AR (irrespective of cellular, antibody or mixed rejection calls) and stable samples with high accuracy (Figure 7A). Most importantly, the CRM expression correlated with molecular inflammation and the extent of graft injury when formulated into a statistically derived CRM-Score using 11/12 defined genes, calculated by using the geometric mean of the expression of each of the CRM genes in each sample. In AR biopsies, the CRM quantitative score correlated with the Banff t-score (p = 6.04e-12) and the Banff i-score (p=2.72e-12) supporting the value of this molecular measurement in the assessment of alloimmune inflammation in the graft. The CRM score in histologically normal 6-month protocol biopsies also correlated strongly with the Banff ct score (p-value = 1.995e-5) and Banff ci score (p-value = 6.195e-7) in transplants that went on to develop progressive chronic allograft injury, versus those that had stable histology and graft function (p=4.9e-6, Figure 7B). Given that histological analysis of the graft can miss subtle changes in inflammation, the inclusion of the CRM score as a surrogate for SCI is a strength of this study in the interpretation of the inflammation response in the kidney and retrospective interrogation for its interplay with other peripheral markers of graft inflammation in the blood and the urine. The finding that the CRM score associates with the extent of graft injury (Figure 7B) as assessed by Banff criteria is of special importance for the current study, as it provides a measure of integrating gene expression profiles with the findings of the immune histopathology core. Moreover, as the CRM score also correlates with an increased risk of subsequent graft fibrosis, it may inform investigators of those at risk for late graft failure and provide mechanistic insight into the success of the proposed interventions.

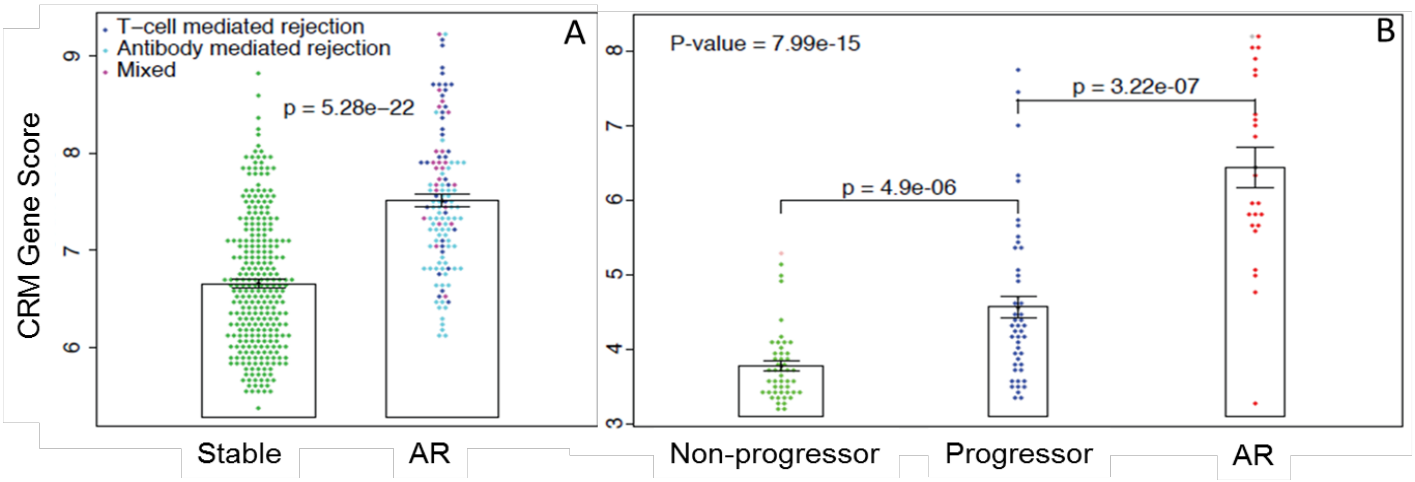


Figure 7:The CRM score significantly correlates with graft function. A. Comparison of CRM score between patients with stable allograft function and acute rejection (AR). **B.** Comparison of CRM in an independent cohort of 120 biopsies (GSE 1563) with histologically and functionally normal renal transplants (Non-progressor), Banff classified progressive chronic allograft injury (Progressor), or Banff graded acute rejection (AR).

Total RNA will be extracted from 3x10¥ìm-thick sections of the FFPE tissue using the PureLink FFPE Total RNA Isolation Kit. RNA quantity and integrity will be determined with the Thermo Scientific NanoDrop ND-2000 UV-Vis Spectrophotometer and Agilent Bioanalyzer, respectively. Gene expression will be analyzed using a set of 800 immune genes that includes the CRM genes. The RNA samples will be added to a Barcoded Codeset designed by NanoString Technologies consisting of Reporter and Capture probes that hybridize to the target sequences of interest, forming a tripartite complex. The raw counts for each assay will be collected using the NanoString data analysis software, nSolver¢ç (NanoString Technologies, Seattle, WA). Normalization of the data will be performed using nSolver¢ç for the following two methods. (1) Positive control normalization: gene expression data is normalized to the mean of the POS control probes for each assay. (2) RNA content normalization: gene expression data is normalized to the geometric mean of housekeeping genes in the CodeSet.

The CRM score is defined as geometric mean of the CRM genes expression. Distribution of CRM scores in AR and STA groups and receiver operating characteristic (ROC) curve are shown for GSE21374 (Figure 8A-B), GSE36059 (Figure 8C-D). The x axes represent false positive rate, and the y axes represents true positive rate when using the CRM scores for predicting AR. Error bars indicate the standard error of the mean (Fig.8). In each independent dataset, the CRM score was significantly higher in the AR group (P < 1.5 ¡¿ 10£¬7; Fig. 8). Each unit increment in the CRM score increased the odds of AR by 4.17 and 5.45 in GSE21374 and GSE36059. It was also able to distinguish AR and STA samples with high specificity and sensitivity in GSE21374 area under the curve (AUC = 0.83; Fig. 8B), GSE36059 (AUC = 0.8; Fig. 8D). The quantitative CRM score will be used to quantify SCI at a molecular level in the 2 week and 7-month protocol biopsies as well as any clinically indicated biopsies in the TASK study. The rest of the ~780 immune genes will also be analyzed along with the CRM genes. The gene expression data will be correlated with the Banff scores and the inflammatory cell load in the biopsies as measured by quantitative image analysis of CD45-stained sections of the FFPE tissue.

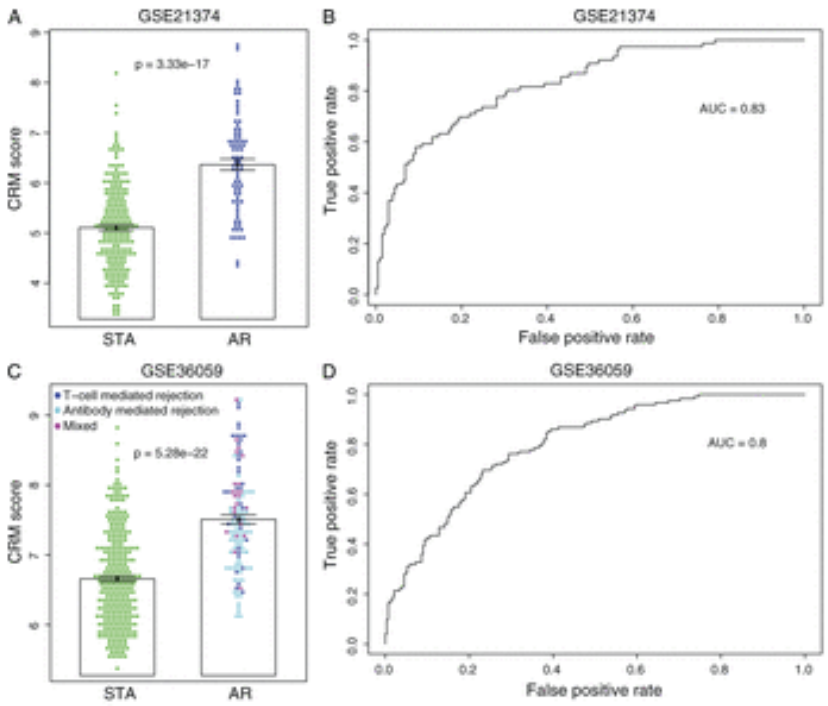


Figure 7. The intragraft CRM gene expression score can distinguish AR and STA samples with high sensitivity and specificity.

11.2.3. Urinary Biomarkers of Graft Inflammation and Injury

Urine protein biomarkers correlate highly with the graft milieu and may directly reflect alloreactive inflammation and injury (Lei J, 2010) (Ling XB, 2010) (Schaub S, 2004) (Schaub S W. J., 2006) (Sigdel TK K. A., 2010). The Sarwal lab has utilized a cohort of over 2000 highly clinically annotated urine samples from ~800 prospectively monitored renal transplant recipients, collected in multiple clinical trials (CCTPT SNSO1 study, U01 AI55795-02; CTOTC IMPACT trial 1U01 AI077821; AARA UO1AI063594-06). By following an unbiased high-throughput 2D-LCMS/MS based proteomic approach, the Sarwal Lab selected 11 urine proteins (fibrinogen β (FBB), fibrinogen γ (FBG), Class II histocompatibility protein, HLA-DRB1 and SUMO2) to develop a composite biomarker panel for transplant injury, distinguishing acute rejection, chronic rejection and BKVN ($p<0.01$; fold increase >1.5). This panel has been validated in 154 independent urine samples, each with biopsy matched phenotypic diagnoses. Interestingly, when histological scores of inflammation from the biopsies matched with the urine sample collections were analyzed, there was a significant correlation ($r=0.73$; $p<0.0003$) between these urinary proteins and mononuclear cell interstitial inflammation score (i-score) and the tubulitis score (t-score), suggesting that these urine proteins in addition to being valuable clinical biomarkers for rejection monitoring, may also segregate biopsies with higher grades of T cell mediated rejection. In addition, mRNA analysis for the CRM genes in urine may also provide a means to monitor for SCI in a non-invasive, serial manner.

Total RNA will be extracted from the urine pellet (Qiagen). Total RNA will be measured for RNA integrity using the RNA 6000 Nano LabChip Kit on a 2100 Bioanalyzer (both from Agilent Technologies, Santa Clara, CA), with suitable RNA defined by an RNA integrity number exceeding 5. Using a urine sample processing method that we developed, urinary proteins will be isolated by filtering the supernatant through Amicon Ultra centrifugal filtration tubes (10K molecular weight cutoff, Millipore, Bedford, MA) to separate small MW peptides and other pigments (<10 kDa) from the larger proteins. Commercially available ELISA assays for the urine protein panels will be purchased from Antibodies-online (Atlanta, GA), and the urine protein levels will be normalized to urine creatinine levels. The relative amount of mRNA expression in each sample will be calculated for each of the CRM genes using the comparative threshold cycle (CT) method. Expression values for all genes will be normalized to ribosomal18S RNA (18S). The levels of specific proteins and

genes will be measured in samples with and without SCI and their abundance also will be correlated with extent of graft fibrosis.

11.3. Impact of Treg Infusion on Peripheral Blood Biomarkers of Rejection and Graft Injury

11.3.1. Peripheral Blood Leukocyte Population and Phenotype by Multiparameter Flow Cytometry (MFC)

Blood (1 ml) will be collected at visits specified in the schedule of events (Appendix 2 and 3), processed into PBMC, and cryopreserved for batched analysis to assess impact of Treg infusion on the frequencies and phenotypes of peripheral blood leukocytes. The MFC analysis is focused on defining leukocyte subsets, T cell activation/exhaustion status, and CD4+ and CD8+ Treg frequencies. Extensively validated MFC panels will be used to quantify changes in leukocyte populations in blood (Table 4) collected prior to and after Treg infusion. The absolute number of each cell types in the peripheral blood will be calculated by referencing to the number of CD4 counts obtained from clinical lab test.

Table 4. TASK MFC Panels

Panel Names	Markers	Rationale
Leuko	CD3, CD4, CD8, CD14, CD16, CD19, CD56, HLA-DR	To determine the numbers and percentages of T cells, B cells, subsets of monocytes, subsets of NKs and dendritic cells
Treg	CD3, CD4, CD8, CD25, CD127, FXOP3, HELIOS	To determine the numbers and percentages of Tregs
Tact	CD3, CD4, CD8, CD27, CD28, CD45RA, CCR7	To determine the activation status of T cells
Texh	CD3, CD4, CD8, CD57, PD1, Tim3	To determine the percentage of T cells that express exhausted or inhibited phenotype

11.3.2. Peripheral Blood Biomarkers of Acute Rejection (kSORT)

Building on a recent publication of the identification of 10 genes in peripheral blood for the diagnosis of pediatric renal allograft rejection in low-risk patients (Rose-John, 2012), an additional 7 genes for the diagnosis and prediction of acute rejection in both pediatric and adult kidney transplant recipients (Roedder S S. T., 2014) was identified, independent of the cause of end stage renal disease, co-morbidities, transplant center or immunosuppression usage. This signature comprises the following genes (CFLAR, DUSP1, ITGAX, RNF130, PSEN1, NKTR, RYBP, NAMPT, MAPK9, IFNGR1, CEACAM4, RHEB, GXMK, RARA, SLC25A37, EPOR, RXRA) which combines the previous 10-gene set in addition to 7 genes obtained through additional inclusion of adult renal transplant data, into the final developed assay that will be used in this study called kSORT (kidney solid organ response test) (Table 5).

The kSORT genes are highly specific and sensitive for the prediction of biopsy confirmed acute rejection at the time of AR, and additionally 3-4 months prior to graft dysfunction and histologically confirmed AR. The 17 genes can be easily used for the prediction of acute rejection using a novel plug-in reference-correlation classification approach called Lineage Profiler; this program has been customized in the Sarwal Lab and calculates an AR risk score where a score >0 indicates AR and a score <0 indicates non-AR (Figure 8). The SORT genes will be analyzed in our trials to define their association with inflammation and borderline rejection and to examine whether they are relevant in the response to therapy with Tregs.

Gene ID	Protein name	Function
DUSP1	Dual specificity protein phosphatase-1	Dephosphorylates MAP kinase to regulate cell cycle
CFLAR	CASP8 and FADD-like apoptosis inhibitor	Cytosolic protein that inhibits tumor necrosis factor related apoptosis
ITGAX	Integrin alpha–X	Membrane receptor for fibrinogen that mediates cell-cell interaction during inflammatory responses; important in monocyte adhesion and chemotaxis
NAMPT	Nicotinamide phosphoribosyltransferase	Catalyzes formation of NAD; also exists as nonsecreted form that acts as cytokine and adipokine; plays a role in regulating circadian clock
MAPK9	Mitogen-activated protein kinase 9	Activated by cytokines and/or stress response to promote cell proliferation, differentiation, migration, transformation and programmed cell death
RNF130	E3 ubiquitin-protein ligase RNF130	Cytoplasmic zinc-binding protein
IFNGR1	Interferon-receptor gamma-1	Membrane protein that binds interferon gamma; mutation causes Immunodeficiency 27A
PSEN1	Presenilin-1	Stimulates cell-cell adhesion; may play role in signaling and apoptosis; mutations associated with familial early-onset Alzheimer's
RYBP	RING1 and YY1-binding protein	Nucleic and cytosolic protein that inhibits transcription, promotes apoptosis
NKTR	NK-tumor recognition protein	Membrane protein that is a component of a putative tumor recognition complex on the surface of NK cells; binds cyclosporin A
SLC25A37	Mitoferrin-1	Mitochondrial iron transporter that takes up iron for developing erythroid cells
CEACAM4	Carcinoembryonic antigen-related cell adhesion molecule 4 (CD66a)	Surface protein found on lymphocytes for cell to cell adhesion
RARA	Retinoic acid receptor alpha	Suppresses transcription when unbound, loss of suppression when bound, associated with spermatocyte survival when bound
RXRA	Rxra protein	Nuclear protein that regulates transcription
EPOR	Erythropoietin receptor	Promotes erythroblast proliferation and differentiation when bound by EPO
GZMK	Granzyme K	Serine protease released by NK or T cells for killing of cancerous cells, viruses, or bacteria
RHEB	GTP-binding protein Rheb	Promotes proliferation and inflammation through activation of mTOR complex

Table 5. kSORT genes

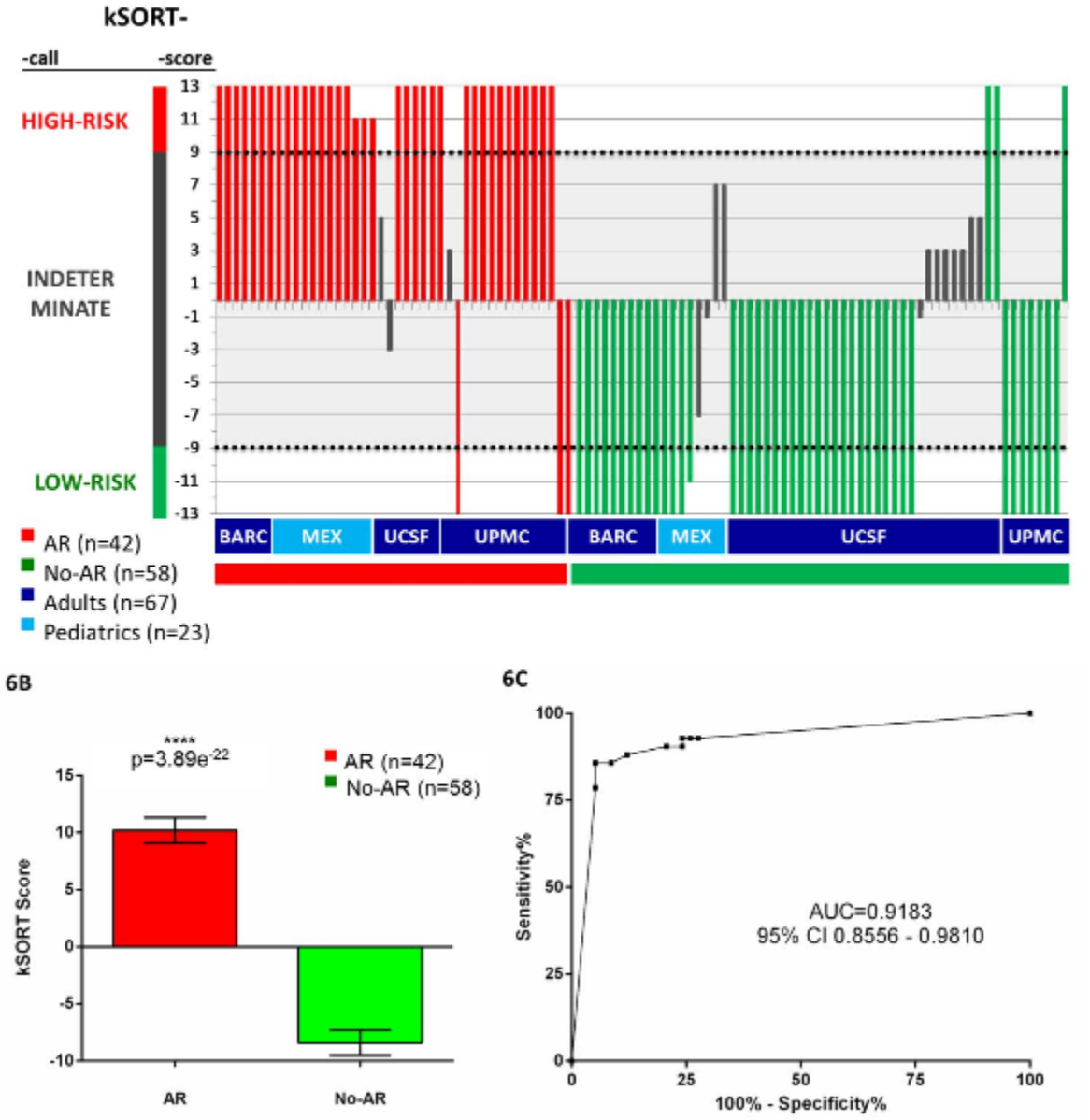


Figure 8. Performance of the kSORT assay. *(A) kSORT score and classification category were calculated for each sample: the kSORT assay correctly classified 36 out of 39 AR samples as high risk for AR (red bars, 92.3%; risk score ≥9) and 43 out of 46 No-AR samples as low risk for AR (green bars, 93.5%, risk score ≤9) across four different sample collection sites (UPMC, Mexico, Barcelona and UCSF), and adult versus pediatric recipient age groups, with samples collected in clinical trial and non-clinical-trial settings; remaining 15 samples classified as indeterminate risk for AR (grey bars; risk score (scores <9 and >-9) (B) Mean aggregated kSORT scores (error bars give standard error of mean) were significantly higher in all true AR samples than in all true No-AR samples by two-sided Student's t test. (C) ROC analysis demonstrated high sensitivity and specificity for the 100 samples evaluated in this dataset.*

Total RNA will be extracted using the column-based method kits of PreAnalytiX (Qiagen). Total RNA will be measured for RNA integrity using the RNA 6000 Nano LabChip Kit on a 2100 Bioanalyzer (both from Agilent Technologies, Santa Clara, CA), with suitable RNA defined by an RNA integrity number exceeding 7. cDNA synthesis will be performed using 250 ng of extracted quality mRNA from the PB samples using the SuperScript II first strand cDNA synthesis kit. Standard protocols developed in the Sarwal Lab will be used for QPCR reactions on the ABI Quant-Studio 6 sing the same TaqMan gene expression assays. The relative amount of mRNA expression in each sample will be calculated for each of the 17 kSORT genes using the comparative threshold cycle (CT) method. Expression values for all genes will be normalized to

ribosomal18S RNA (18S). All samples will be evaluated against our customized database (kSAS) of centroids for AR and No-AR, with the greatest correlation to the evaluated sample. Classification of a sample as AR, No-AR, or indeterminate will be derived by computing the Pearson correlation coefficients for all sample–centroid comparisons, assigning a score of 1 to samples with a greater correlation to an AR centroid and a score of -1 to samples with a greater correlation to a No-AR centroid. A kSAS aggregated AR risk score (-13 to 13) will then be determined by summing the scores for all evaluated gene models. Indeterminate samples will be defined as those with a score less than 9 and greater than -9, based on evaluation of sample classification. The blood sample kSORT scores will be correlated with SCI and clinical AR (expected kSORT scores of ≥9) as well as stable, quiescent samples (expected kSORT scores of ≥ -9) in the TASK study.

11.3.3. Alloimune Quiescence and Operational Tolerance (kSPOT)

Sarwal Lab has identified an initial tolerance footprint of 49 genes by microarray and qPCR in peripheral blood samples from 75 renal allograft recipients and 16 healthy individuals from the U.S. and in Europe (Brouard S, 2007). 21/49 genes correctly segregated tolerance and chronic rejection phenotypes with 99% and 86% specificity, and the signature was shared with 8% and 50% respectively of stable patients on triple immunosuppression and low-dose steroid monotherapy respectively. The gene signature suggested a pattern of reduced co-stimulatory signaling, immune quiescence, apoptosis, and memory T cell responses, and additionally suggested that TGFβ might contribute to this process. This panel has been reduced to a minimal 3-gene signature (BNC2, CYP1B1, KLF6) called SPOT (Spontaneous Tolerance Test) using penalized logistic regression modeling. The inclusion of SPOT in the proposed trials will therefore provide an assessment of immune quiescence that may then be deployed as a measure for the success of Treg therapy. Additionally, these results provide a proof of concept for successfully linking the gene expression profiles in peripheral blood with the peripheral blood phenotype measured by flow cytometry.

Sample collection and analysis plan for kSPOT. Please refer to kSORT for all sample collection and processing. The 21 gene logistic regression score for a tolerance like signature (Roedder S, 2014) will determine immune quiescence profile for all samples in the TASK study and will evaluate if this signature is more likely to be found in patients who have received Treg therapy.

11.3.4. Non-HLA Antibody Assays for Prediction of Chronic Allograft Injury (nHLA-Ab)

The Sarwal Lab has identified and validated a de novo panel of 6 nHLA- Ab (MIG, ITAC, IFN-γ, GABPA, IL8, CCL21, GDNF) at 6 months post-transplant as important and previously unrecognized triggers of subsequent chronic graft injury (Sigdel TK, 2012). This panel positively correlated ($r=0.78$; $p<0.01$) with the chronic allograft damage index (Yilmaz S, 2003) scores at 2 years with 80% sensitivity and 100% specificity. This discovery involved the use of high-throughput screening of serum IgG interactions in post-transplant recipient sera to 9000 unique full-length human proteins using the Protoarray (Invitrogen) protein array technology and data analysis by customized algorithms (Li L, 2009). The Sarwal Lab has generated customized reverse ELISA assays for this panel of nHLA-Ab to be easily multiplexed on the Meso Scale Discovery® (MSD) platform. These specific nHLA-Ab panels will be correlated with histological chronicity and change in eGFR in the different treatment arms of the study.

Sample processing analysis plan for nHLA MSD ELISA will be carried out following the protocol developed in the Sarwal lab. Briefly 15 ng antigen will be coated on to the wells using 50 µL coating at 0.3 µg/ml. The plates will be stored at 4 °C overnight. The following day the coating solution is removed and the plate is blocked by 150 µl Blocker A (at RT, 450 rmp) for 1 hr. After 1 hr the plate will be washed 3 times with 300 µl wash buffer. Next, 25 µl of diluted samples will be added and incubated at RT with 450 rpm for 2 hr. This will be followed by washing step followed by addition of 25 µl detection antibody SULFO-TAG Goat anti Human (Meso Scale Discovery, Gaithersburg MD, Cat# R32AJ-5) (1:1000 dilution) for 1 hr. After this step the plates will be washed and 150ul/well of 1X read buffer (MSD Gaithersburg MD, Cat#

R92TC-2) will be added to each well. The plates will be read immediately using MESO QuickPlex SQ 120 scanner. Relative signal intensity will be used to measure relative abundance of the nHLA antibodies in the samples. The data will be analyzed by Mann-Whitney test using GraphPad Prism software. Spearman correlation will be done to correlate nHLA Ab signal with histological chronicity and change in eGFR in the different treatment arms of the study. These specific nHLA-Ab panels will be correlated with histological chronicity and change in eGFR in the different treatment arms of the study.

11.3.5. Markers of Tubular Epithelial Injury and Renal Fibrosis

Urine and serum markers of renal injury have been associated with subsequent graft fibrosis and failure. Preliminary studies in Dr. Mannon's laboratory have identified urinary TGFβ, connective tissue growth factor (CTGF) and the integrin ligand vitronectin as associated with allograft injury and fibrosis (Cheng O, 2008) (Mannon RB, 2010). In addition, urinary ANXA11, α3 and β3 integrins and TNF-α have been found to be closely associated with acute alloimmune mediated injury in kidney transplant recipients (Srivastava M, 2011) but have not yet been analyzed prospectively. A novel link under study is between innate immune activation and allograft fibrosis, and measures of HMGB1 and HSP 27 in serum and urine will be of value (Harris HE, 2012) (Lotze M, 2005). The recent association of resistin with brain death and delayed graft function (Oltean S, 2013), as well as the association of acute inflammation, endothelial activation and fibrosis (Jackson AM, 2014) suggest this as a protein that can be measured in this prospective cohort. Having such targets is critical while investigating therapies which may mitigate inflammatory injury that leads to subsequent graft failure in order to monitor the success or failure of treatment. Other relevant targets for monitoring include expression of matrix molecules such as collagens I and III (Mannon R, 1999)and fibroblast infiltration such as α-SMA as indicators of developing fibrosis (Cheng O, 2008) as well as recently described markers of myeloid fibroblast infiltration including MCP-1, CXCL16 and adiponectin (Yang J, 2013). Establishing a link between these markers and graft histology/ renal function is a unique feature of this proposal in which protocol biopsies provide a "hard endpoint" in the subjects' management.

11.4. Stored Plasma and Serum

Plasma and serum will be collected on the day of planned infusion, prior to polyTreg administration for subjects in Group 2 . If a need arises during the course of the study, testing may be performed on archived specimens (e.g. CMV, BK, DSA etc.).

12. Biospecimen Storage

Biological specimens (i.e., whole blood, plasma, serum, urine supernatant, urine pellet) obtained under this protocol may be used in future assays to reevaluate biological responses as additional research tests are developed over time. Residual specimens collected at time points planned for the core mechanistic studies will be maintained to allow specimens to be stored for possible use in new assays that have yet to be optimized or conceived, or assays performed by other CTOT members for cross-validation studies. Appropriate informed consent will be obtained for both the collection and storing of samples. During the funding period, samples will be identifiable, which means samples will be coded with a subject ID number that could be directly linked to the subject and the subject's medical record. The specimens from these evaluations may be stored beyond the funding period. Samples will be identifiable, which means samples will be coded with a subject ID number that could be directly linked to the subject and the subject's medical record. When the funding period is over, data from the clinical database will be de-identified, meaning patient identifiers will be removed, and all associated date information will be transformed to study days. Core laboratories and/or repositories maintaining specimens will not have the ability to identify a subject or access data associated with a specimen without specific approval from the study team.

13. Criteria for Participant Completion and Premature Study Termination

13.1. Participant Stopping Rules and Withdrawal Criteria

Participants may be prematurely terminated from the study for the following reasons:

1. The participant elects to withdraw consent from all future study activities, including follow-up.
2. The participant is "lost to follow-up" 3 months after the date of a missed study visit (i.e., no further follow-up is possible because attempts to reestablish contact with the participant have failed).
3. The participant dies.
4. The Investigator no longer believes participation is in the best interest of the participant.
5. CTCAE Grade 3 or higher infusion reaction.
6. Failure to manufacture and supply the cellular product two times for the same subject.

13.2. Participant Replacement

Participants who are prematurely terminated from this study following infusion of at least 100 x 10^6 Tregs will not be replaced. Subjects who are prematurely terminated prior to polyTreg infusion will be replaced.

13.2.1. Follow-up after Early Study Withdrawal

If a participant in Group 2 is withdrawn from the study for any reason, the participant will be asked to complete a final visit for safety assessment at 52 weeks after polyTregs (Day 364/Visit 6 on Appendix 3).

13.3. Study Stopping Rules

The study may be prematurely terminated for the following reasons:

1. Any CTCAE grade 4 or higher infusion reaction
2. Any study defined grade 4 or higher infection
3. Any diagnosis of malignancy and post-transplant lymphoproliferative disease (except non-melanoma skin cancer)
4. Any graft loss
5. Acute Rejection (Banff grade 2A or higher) or acute antibody mediated rejection within 6 weeks after Treg infusion in 3 of first 5 subjects
6. Any death
7. Inability to manufacture and supply polyTregs in 3 of 5 consecutive subjects assigned to Group 2
8. If there is a total of 8 failed lots of polyTregs at any time during the study.

Any of these events will stop trial enrollment and Treg infusions and require DSMB review (See Section 14.8). Subjects who have already received Treg infusion are eligible for mTOR therapy will remain on current therapy during the data review.

14. Safety Monitoring and Reporting

14.1. Overview

This section defines the types of safety data that will be collected under this protocol and outlines the procedures for appropriately collecting, grading, recording, and reporting those data. Adverse events that are classified as serious according to the definition of health authorities must be reported promptly to the sponsor *[DAIT/NIAID]*. Appropriate notifications will also be made to site principal investigators, Institutional Review Boards (IRBs), and health authorities.

Information in this section complies with ICH Guideline E2A: Clinical Safety Data Management: Definitions and Standards for Expedited Reporting, ICH Guideline E-6: Guideline for Good Clinical Practice, 21CFR Parts 312 and 320, and applies the standards set forth in the National Cancer Institute (NCI), Common Terminology Criteria for Adverse Events (CTCAE), Version 4.0: http://ctep.cancer.gov/reporting/ctc.html.

14.2. Definitions

14.2.1. Adverse Event (AE)

Any untoward or unfavorable medical occurrence associated with the subject's participation in the research, whether or not considered related to the subject's participation in the research (modified from the definition of adverse events in the 1996 International Conference on Harmonization E-6 Guidelines for Good Clinical Practice) (from OHRP "Guidance on Reviewing and Reporting Unanticipated Problems Involving Risks to Subjects or Others and Adverse Events (1/15/07)" http://www.hhs.gov/ohrp/policy/advevntguid.html#Q2)

14.2.1.1. *Suspected Adverse Reaction (SAR)*

Any adverse event for which there is a reasonable possibility that the investigational drug [or investigational study therapy regimen] caused the adverse event. For the purposes of safety reporting, 'reasonable possibility' means there is evidence to suggest a causal relationship between the drug and the adverse event. A suspected adverse reaction implies a lesser degree of certainty about causality than adverse reaction, which means any adverse event caused by a drug (21 CFR 312.32(a)).

14.2.2. Unexpected Adverse Event

An adverse event or suspected adverse reaction is considered "unexpected" if it is not listed in the Investigator Brochure or package insert or is not listed at the specificity, severity or rate of occurrence that has been observed.

"Unexpected" also refers to adverse events or suspected adverse reactions that are mentioned in the Investigator Brochure or package insert as occurring with a class of drugs or as anticipated from the pharmacological properties of the drug, but are not specifically mentioned as occurring with the particular drug under investigation (21 CFR 312.32(a)*]*

14.2.3. Serious Adverse Event (SAE)

An adverse event or suspected adverse reaction is considered "serious" if, in the view of either the investigator or Sponsor, it results in any of the following outcomes (21 CFR 312.32(a)):

1. Death.
2. A life-threatening event: An AE or SAR is considered "life-threatening" if, in the view of either the investigator or Sponsor [add DAIT/NIAID or other Sponsor, *if applicable],* its occurrence places the subject at immediate risk of death. It does not include an AE or SAR that, had it occurred in a more severe form, might have caused death.
3. Inpatient hospitalization or prolongation of existing hospitalization.

4. Persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions.
5. Congenital anomaly or birth defect.
6. Important medical events that may not result in death, be life threatening, or require hospitalization may be considered serious when, based upon appropriate medical judgment, they may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed above.

Elective hospitalizations or hospital admissions for the purpose of conduct of protocol mandated procedures are not to be reported as an SAE unless hospitalization is prolonged due to complications.

14.2.4. Other Significant Adverse Events

The events below should be reported (entered on the AE/SAE form) within 24 hours of awareness even if the event does not meet serious criteria:

- Biopsy proven or clinical (Treated) AR
- Chronic rejection
- Infusion Reactions (CTCAE grade 2 or higher)
- Malignancy, PTLD
- Infections (study defined grade 3 or higher)
- COVID-19 infections of any grade

If no other serious criteria are met for COVID-19 infection, the event should be considered of medical importance.

14.3. Grading and Attribution of Adverse Events

14.3.1. Grading Criteria

The study site will grade the severity of adverse events experienced by the study subjects according to the criteria set forth in the National Cancer Institute's Common Terminology Criteria for Adverse Events (CTCAE) *version 4.0.*

This document (referred to herein as the NCI-CTCAE manual) provides a common language to describe levels of severity, to analyze and interpret data, and to articulate the clinical significance of all adverse events. The NCI-CTCAE has been reviewed by the principal investigator and protocol chair and has been deemed appropriate for the subject population to be studied in this protocol.

Infections will be graded using the study-specific scale as described below:

Grade 1 = asymptomatic; clinical or diagnostic observation only; intervention with oral antibiotic, antifungal, or antiviral agent only; no invasive intervention required

Grade 2 = symptomatic; intervention with intravenous antibiotic, antifungal, or antiviral agent; invasive intervention may be required

Grade 3 = any infection associated with hemodynamic compromise requiring pressors; any infection necessitating ICU level of care; any infection necessitating operative intervention; any infection involving the central nervous system; any infection with a positive fungal blood culture; any proven or probable aspergillus infection; any tissue invasive fungal infection; any pneumocystis jiroveci infection

Grade 4 = life-threatening infection

Grade 5 = death resulting from infection

All other adverse events will be graded on a scale from 1 to 5 according to the following standards in the NCI-CTCAE manual:

Grade 1 = mild adverse event.

Grade 2 = moderate adverse event.

Grade 3 = severe and undesirable adverse event.

Grade 4 = life-threatening or disabling adverse event.

Grade 5 = death.

Infection events grade 2 or higher; and all other events CTCAE grade 2 or higher will be recorded on the appropriate AE eCRF for this study.

For grading an abnormal value or result of a clinical or laboratory evaluation (including, but not limited to, a radiograph, an ultrasound, an electrocardiogram etc.), a treatment-emergent adverse event is defined as an increase in grade from baseline or from the last post-baseline value that doesn't meet grading criteria. Changes in grade from screening to baseline will also be recorded as adverse events but are not treatment-emergent. If a specific event or result from a given clinical or laboratory evaluation is not included in the NCI-CTCAE manual, then an abnormal result would be considered an adverse event if changes in therapy or monitoring are implemented as a result of the event/result.

14.3.2. Attribution Definitions

The relationship, or attribution, of an adverse event to the study therapy regimen or study procedure(s) will initially be determined by the site investigator and recorded on the appropriate AE/SAE eCRF. Final determination of attribution for safety reporting will be determined by DAIT/NIAID. The relationship of an adverse event to study therapy regimen or procedures will be determined using the descriptors and definitions provided in Table 6.

For additional information and a printable version of the NCI-CTCAE manual, consult the NCI-CTCAE web site: http://ctep.cancer.gov/reporting/ctc.html.

Table 6. Attribution of Adverse Events

Code	Descriptor	Relationship (to primary investigational product and/or other concurrent mandated study therapy or study procedure)
Unrelated Category		
1	Unrelated	The adverse event is clearly not related: there is insufficient evidence to suggest a causal relationship.
Related Categories		
2	Possible	The adverse event has a reasonable possibility to be related; there is evidence to suggest a causal relationship.
3	Related	The adverse event is clearly related.

Attribution of adverse event to tacrolimus, MMF/MPA, and prednisone will not be assessed in this study because these medications are used as standard of care for kidney transplant recipients.

Attribution assessment for the following study interventions and procedures will be made when a SAE is reported:

Study therapy regimen:

1. polyTregs

Study mandated procedures:

1. Leukapheresis
2. Kidney biopsy
3. Blood Draw (Donor or Recipient)
4. IS regimen conversion / Everolimus

14.4. Collection and Recording of Adverse Events

14.4.1. Collection Period

Adverse events will be collected from the time of first study mandated procedure until a subject completes study participation or until 30 days after he/she prematurely withdraws (without withdrawing consent) or is withdrawn from the study.

Note that the study eligibility is performed as a standard of care surveillance biopsy with no collection of research specimens. The eligibility biopsy is not considered a study mandated procedure for these reasons.

14.4.2. Collecting Adverse Events

Adverse events (including SAEs) may be discovered through any of these methods:

- Observing the subject.
- Interviewing the subject [e.g., using a checklist, structured questioning, diary, etc.] .
- Receiving an unsolicited complaint from the subject.
- In addition, an abnormal value or result from a clinical or laboratory evaluation can also indicate an adverse event, as defined in Section 14.2.4, *Grading and Attribution of Adverse Events.*

14.4.3. Recording Adverse Events

Throughout the study, the investigator will record adverse events and serious adverse events as described previously (Section 14.2, *Definitions)* on the appropriate AE/SAE eCRF regardless of the relationship to study therapy regimen or study procedure.

Once recorded, an AE/SAE will be followed until it resolves with or without sequelae, or until the end of study participation, or until 30 days after the subject prematurely withdraws (without withdrawing consent)/or is withdrawn from the study, whichever occurs first.

14.5. Reporting of Serious Adverse Events and Adverse Events

14.5.1. Reporting of Serious Adverse Events to Sponsor

This section describes the responsibilities of the site investigator to report serious adverse events to the sponsor via the AE/SAE eCRF. Timely reporting of adverse events is required by 21 CFR and ICH E6 guidelines.

Site investigators will report all serious adverse events (see Section 14.2.3, *Serious Adverse Event)*, regardless of relationship or expectedness within 24 hours of discovering the event.

For serious adverse events, all requested information on the AE/SAE eCRF will be provided. However, unavailable details of the event will not delay submission of the known information. As additional details become available, the AE/SAE eCRF will be updated and submitted.

14.5.2. Reporting to Health Authority

After an adverse event requiring 24-hour reporting (per Section 14.5.1, *Reporting of Serious Adverse Events to Sponsor*) is submitted by the site investigator and assessed by DAIT/NIAID, there are two options to report the adverse event to the appropriate health authorities:

14.5.2.1 Annual Reporting

DAIT/NIAID will include in the annual study report to health authorities all adverse events classified as:

- Serious, expected, suspected adverse reactions (see Section 14.2.1.1, *Suspected Adverse Reaction,* and Section 14.2.2, *Unexpected Adverse Event*).
- Serious and not a suspected adverse reaction (see Section 14.2.1.1, *Suspected Adverse Reaction*).
- Pregnancies.

Note that all adverse events (not just those requiring 24-hour reporting) will be reported in the Annual IND Report.

14.5.2.2 Expedited Safety Reporting

DAIT/NIAID shall notify the FDA and all participating investigators of expedited Safety Reports within 15 calendar days; unexpected fatal or immediately life-threatening suspected adverse reaction(s) shall be reported as soon as possible or within 7 calendar days.

This option, with 2 possible categories, applies if the adverse event is classified as one of the following:

Category 1: **Serious and unexpected suspected adverse reaction** [**SUSAR]** (see Section 14.2.1.1, *Suspected Adverse Reaction* and Section 14.2.2, *Unexpected Adverse Event* and 21 CFR 312.32(c)(1)i).

The sponsor shall report any suspected adverse reaction that is both serious and unexpected. The sponsor shall report an adverse event as a suspected adverse reaction only if there is evidence to suggest a causal relationship between the study drug and the adverse event, such as:

1. A single occurrence of an event that is uncommon and known to be strongly associated with drug exposure (e.g., angioedema, hepatic injury, or Stevens-Johnson Syndrome);
2. One or more occurrences of an event that is not commonly associated with drug exposure, but is otherwise uncommon in the population exposed to the drug (e.g., tendon rupture);
3. An aggregate analysis of specific events observed in a clinical trial (such as known consequences of the underlying disease or condition under investigation or other events that commonly occur in the study population independent of drug therapy) that indicates those events occur more frequently in the drug treatment group than in a concurrent or historical control group.

Category 2: Any findings from studies that suggests a significant human risk

The sponsor shall report any findings from other epidemiological studies, analyses of adverse events within the current study or pooled analysis across clinical studies or animal or *in vitro* testing (e.g., mutagenicity, teratogenicity, carcinogenicity) that suggest a significant risk in humans exposed to the drug that would result in a safety-related change in the protocol, informed consent, investigator brochure or package insert or other aspects of the overall conduct of the study.

14.5.3. Reporting of Adverse Events to IRBs/IECs

All investigators shall report adverse events, including expedited reports, in a timely fashion to their respective IRBs/IECs in accordance with applicable regulations and guidelines. All Safety Reports to the FDA shall be distributed by DAIT/NIAID or designee to all participating institutions for site IRB/IEC submission.

14.6. Pregnancy Reporting

The investigator shall be informed immediately of any pregnancy in a study subject or a partner of a study subject. A pregnant subject shall not receive Tregs, and will be instructed to stop taking everolimus, if applicable. The investigator shall counsel the subject and discuss the risks of continuing with the pregnancy and the possible effects on the fetus. Monitoring of the pregnant subject shall continue until the conclusion of the pregnancy.

The investigator shall report to the Statistical and Clinical Coordinating Center (SACCC) all pregnancies within 1 business day of becoming aware of the event using the Pregnancy eCRF. All pregnancies identified during the study shall be followed to conclusion and the outcome of each must be reported. The Pregnancy eCRF shall be updated and submitted to the DAIT/NIAID via the SACCC when details about the outcome are available. When possible, similar information shall be obtained for a pregnancy occurring in a partner of a study subject.

Information requested about the delivery shall include:

1. Gestational age at delivery
2. Birth weight, length, and head circumference
3. Gender
4. Appearance, pulse, grimace, activity, and respiration (APGAR) score at 1 minute, 5 minutes, and 24 hours after birth, if available
5. Any abnormalities.

All pregnancy complications that result in a congenital abnormality, birth defect, miscarriage, and medically indicated abortion - an SAE shall be submitted to the DAIT/NIAID via the SACCC using the SAE reporting procedures described above. Pregnancies reported as SAE's will be reported to the FDA as described above.

14.7. Reporting of Other Safety Information

An investigator shall promptly notify the site IRB as well as the SACCC using the AE/SAE eCRF when an "unanticipated problem involving risks to subjects or others" is identified, which is not otherwise reportable as an adverse event.

14.8. Review of Safety Information

14.8.1. Medical Monitor Review

The PI, Protocol Chair, and NIAID Medical Monitor will review safety data on an ongoing basis. Enrollment and initiation of study treatment may be suspended at any time if these reviews conclude that there are significant safety concerns.

In addition, the Medical Monitor shall review and make decisions on the disposition of the SAE and pregnancy reports received by the SACCC in a timely manner.

14.8.2. Planned DSMB Reviews

The Data and Safety Monitoring Board (DSMB) shall review safety data at least yearly during planned DSMB Data Review Meetings. Data for the planned safety reviews will include, at a minimum, a listing of all reported AEs and SAEs.

The DSMB will be informed of an Expedited Safety Report in a timely manner.

14.8.3. *Ad* hoc DSMB Reviews

In addition to the pre-scheduled data reviews and planned safety monitoring, the DSMB may be called upon for *ad hoc* reviews. The DSMB will review any event that potentially impacts safety at the request of the PI, protocol chair or DAIT/NIAID. After review of the data, the DSMB will make recommendations regarding study conduct and/or continuation.

15. Statistical Considerations and Analytical Plan

15.1. Overview

CTOT-21 is a Phase 2 open label study, in which 14 adult kidney transplant recipients will be allocated to one of 2 treatment arms: CNI-only, or polyTregs. Subjects allocated to the Treg treatment arm will receive a single infusion of polyTregs approximately 7 months after transplant. The primary safety objective is to evaluate the safety of polyTregs in adult kidney transplant recipients. The primary efficacy objective of the study is to evaluate whether polyTregs reduce graft inflammation when compared to CNI-based maintenance therapy.

15.2. Measures to Minimize Bias

The study groups will be assigned in an un-blinded fashion using a variation of the Pocock and Simon adaptive randomization algorithm designed to maintain an overall 1:1 ratio. Centralized laboratories will be used to minimize bias and mechanistic core laboratory personnel, including the core pathologist, will be blinded to study treatment assignments. Clinical sites will not be blinded to treatment assignments and reports for site use and monitoring purposes will not be blinded.

15.3. Analysis Plan

Statistical analyses of the safety and clinical outcomes will be performed for the analysis samples defined below in section 15.3.1, employing standard methods for the estimation of incidence rates and their exact two-sided 95% confidence intervals. Statistical analyses of most mechanistic outcomes will be exploratory in nature. The subject enrolled and treated in the darTreg arm, prior to the removal of that treatment arm in protocol version 9.0, will be individually summarized, including but not limited to adverse events, infusion details, and inflammation scores and changes. The plans for statistical analyses of study data will be described in more detail in a Statistical Analysis Plan (SAP).

15.3.1. Analysis Samples

The statistical analyses will be performed on the following subject samples:

1. The Modified Intent-to-Treat Sample 1 (mITT1) consists of all subjects allocated to the CNI-based treatment regimen or received polyTregs while on study.
2. The Per Protocol Sample (PP) consists of all subjects allocated to the CNI-based treatment regimen or received at least 300 x 10^6 of polyTregs while on study that did not have major protocol deviations of concern.
3. The modified intent-to-Treat Sample 2 (mITT2) consists of all subjects who received polyTregs (Group 2).

15.3.2. Endpoint Assessments

15.3.2.1 Primary and Secondary Safety Endpoints

Table 7. Analyses of Safety Endpoints describes the safety endpoints and their corresponding parameters to be estimated in the study. Safety endpoints will be listed or summarized, as appropriate, using standard descriptive statistics for continuous and categorical data.

Table 7. Analyses of Safety Endpoints

ENDPOINT	DESCRIPTIVE PARAMETER	ANALYSIS POPULATION
Primary Endpoint		
Incidence of severe acute rejection or antibody mediated rejection in subjects receiving polyTregs in comparison with CNI-based maintenance IS therapy.	Proportion with exact binomial 95% confidence limits	mITT1
Incidence of severe infection in subjects receiving polyTregs in comparison with CNI-based maintenance IS therapy.	Proportion with exact binomial 95% confidence limits	mITT1
Secondary Endpoints – Treg safety		
Incidence of polyTregs infusion reactions (episodes of fever, bronchospasm, hypoxia, cytokine release syndrome, or infusion site reaction)	Proportion with exact binomial 95% confidence limits	mITT2
Severity of polyTregs infusion reactions	Descriptive CTCAE categories	mITT2
Incidence of culture-proven and clinically diagnosed infections	Proportion with exact binomial 95% confidence limits	mITT1
Severity of culture-proven and clinically diagnosed infections	Descriptive CTCAE categories	mITT2
Incidence of severe infection in subjects receiving polyTregs in comparison with CNI-based maintenance IS therapy.	Proportion with exact binomial 95% confidence limits	mITT1
Timing of acute rejection	Mean time to rejection and standard deviation	mITT1
Incidence of acute rejection	Proportion with exact binomial 95% confidence limits	mITT1
Severity of acute rejection	Descriptive Banff criteria	mITT1
Incidence of CMV reactivation	Proportion with exact binomial 95% confidence limits	mITT1
Incidence of BK viremia	Proportion with exact binomial 95% confidence limits	mITT1
Incidence of >10% decrease in eGFR from baseline	Proportion with exact binomial 95% confidence limits	mITT1
Secondary Endpoints – mTOR therapy		
Incidence of acute rejection	Proportion with exact binomial 95% confidence limits	mITT2 (Treg mTOR vs Treg no mTOR)

15.3.2.2 Supportive Analyses of the Primary Safety Endpoints

Although the study is not powered to detect clinically significant differences in safety event incidence between treatment groups, in a supportive analysis, the incidence of the composite endpoint will be compared between the two treatment groups by an overall Fisher's exact test using the mITT1 analysis sample to compare the incidence between the two groups.

15.3.2.3 Primary Efficacy Endpoint

The primary efficacy endpoint is the mean change from baseline in percent inflammation on biopsy at 7 months and it will be compared between the two treatment groups by ANOVA using the mITT1 analysis sample. The primary endpoint data will be summarized in the 2 groups using boxplots, along with means and 95% confidence intervals.

15.3.2.4 Secondary Efficacy Endpoints

Table 8 describes secondary efficacy endpoints and their corresponding parameters to be estimated in the study. Efficacy endpoints will be listed or summarized, as appropriate, using standard descriptive statistics for continuous and categorical data, including confidence intervals. All secondary endpoints will be descriptive in nature due to the small sample size.

Table 8. Analyses of Secondary Efficacy Endpoints

SECONDARY ENDPOINT	DESCRIPTIVE PARAMETER	ANALYSIS SAMPLE*
Number of subjects who exhibit a relative decrease of 25% or more inflammation on kidney biopsy 7 months after study group allocation compared to baseline	Proportion with exact binomial 95% confidence limits	mITT1
Number of subjects who exhibit a relative decrease of 25% or more inflammation on kidney biopsy 2 weeks after Treg infusion compared to baseline	Proportion with exact binomial 95% confidence limits	mITT2
Number of subjects who exhibit a relative decrease of 50% or more inflammation on kidney biopsy 2 weeks after Treg infusion compared to baseline	Proportion with exact binomial 95% confidence limits	mITT2

15.3.2.5 Supportive Analyses of the Primary Efficacy Endpoints

In addition to the primary efficacy endpoint analysis described above, several supportive analyses will be performed:

1. Since the occurrence of treated rejection prior to or at the time of the 7 month biopsy may confound the measurement of inflammation, the ANOVA described above will be repeated deleting any subjects from the analysis who had treated rejection prior to the 7 month biopsy.
2. Since clinical site, donor type, and race may confound the interpretation of the primary analysis, ANCOVA will be used to separately test for the effect of site, donor type, and race on the primary endpoint analysis.
3. If the PP sample differs from the mITT1 sample, the primary endpoint analysis will be repeated on the PP analysis sample.

15.3.2.6 Mechanistic Endpoints

Three of the mechanistic assays, CRM, SORT, and DEU, which have high level of sensitivity and specificity, will be used to evaluate mechanistic efficacy endpoints (Table 9).

Table 9. Analysis of mechanistic endpoints

Endpoints	Assays	Measurements
Pharmacokinetics of infused Tregs in blood and graft	**DEU¶**	% deuterium enrichment in blood and graft
Rejection gene in blood	**SORT¶**	Pearson's Correlation Coefficient based Z score; $Z = \chi - \mu_{global} / \sigma_{global}$
Inflammatory gene in graft	**CRM¶**	Geometric mean score of the expression of 11 of the 12 CRM genes in each sample

¶ DEU, SORT and CRM assays are for evaluation of efficacy endpoints

15.3.2.7 Analyses of Exploratory Endpoints

Planned mechanistic assays other than CRM, SORT, and DEU are for exploratory assessment of the impact of specific therapies on inflammation and borderline rejection. Any statistically significant results from these tests will be reported as requiring independent verification and will provide a basis for further studies.

Treatment groups and comparators: We will analyze the readouts on assays longitudinally. Patient measurements will be obtained before and after treatment. There will be at least 2 time points recorded for the TASK trial (before and after Treg infusion). The primary interest of analysis is to compare longitudinal changes in assays between different groups: SCI with SOC, and SCI with polyTregs. Two groups of patients, those with normal histology and those with acute rejection on the 6-month surveillance biopsy, will serve as negative and positive controls respectively for the mechanistic studies. We expect the changes over time in these assays to be approximately linear. If after examination of the data appears to not be the case then we will either transform the assay measures or model the time axis time points as an unordered factor (similar to repeated measures ANOVA, but without the requirement for fully balanced data).

Table 10. Analysis of exploratory mechanistic endpoints

Endpoints	Assays	Measurements
Total Tregs in blood and graft	MFC	% and absolute numbers of Tregs
	mIHC	% FOXP3+ among CD3+ cells
Infused Tregs in blood and graft	TCR*	# and frequency of clones from Treg product
Cellular alloimmune profile	MFC	% and absolute numbers of various cell subsets
	SUPP	% suppression at 1 Treg:4 PBMC ratio
Cellular viral immune profile	VIR	Frequencies of CMV and EBV reactive CD8 T cells; % polyfunctional anti-viral T cells
Antibody responses	HLA	DSA+ or DSA-; MFI if DSA+
	nHLA	Concentration of antibodies to 6 nHLA antigens
Immune tolerance gene in blood	SPOT	Penalized Logistic Regression score for 3 genes
Inflammation in graft	HIS	% inflammatory cell load by area; % area with interstitial fibrosis
IL6 signaling in the graft	mIF ISH§	+cells /high power field; number of ISH dots/high power field
Graft fibrosis	FIB	Concentration of protein associated with tubular injury; concentration of pro-fibrotic proteins
	uFIB	Concentration of protein associated with fibrosis
Inflammation markers in urine	uPRO	Concentration of proteins associated with kidney injury and inflammation in urine
	uRNA	Absolute copy number of mRNA for genes associated with acute rejection

15.3.3. Descriptive Analyses

We will use descriptive analyses to summarize subject characteristics of our study populations across treatment groups. Dichotomous variables will be summarized as proportions with 95% confidence intervals. Continuous variables will be summarized using means, standard deviations, and 95% confidence intervals if they are symmetric and unimodal. Otherwise, they will be summarized using the median and the interquartile range. Simple t-, chi-squared, or Fisher's exact test, as appropriate, will be used to compare quantitative measures across treatment groups. The following variables will be summarized:

1. baseline and demographic characteristics
2. use of concomitant medications
3. reasons for early termination
4. all reported AEs

15.4. Interim Analyses

No formal interim analyses of this study are planned.

15.5. Sample Size Considerations

No formal sample size calculations will be performed on the primary safety endpoint as it is descriptive in nature. However, sample sizes calculations were done based on the primary efficacy endpoint. There is no previous data to guide the estimation of treatment effect size and variance for the assessment of clinical efficacy. For the primary efficacy endpoint analysis, which is the mean reduction in inflammation from baseline at 7 months after study group allocation in the mITT1 analysis sample compared between the 2 groups, we have used a two sample 2-sided t-test to determine the estimated power for differences in mean reduction between the treatment groups (ranging from 10-50% reduction in inflammation) at different standard deviations (SD) of the mean change in inflammation (ranging from10-30%). These power estimates are shown in the following Figure 9 and

Table 10. Analysis of exploratory mechanistic endpoints for sample size of 7 in each group and an alpha level of 0.05 and 2-tailed tests.

Figure 9. Revised Sample Size Calculations

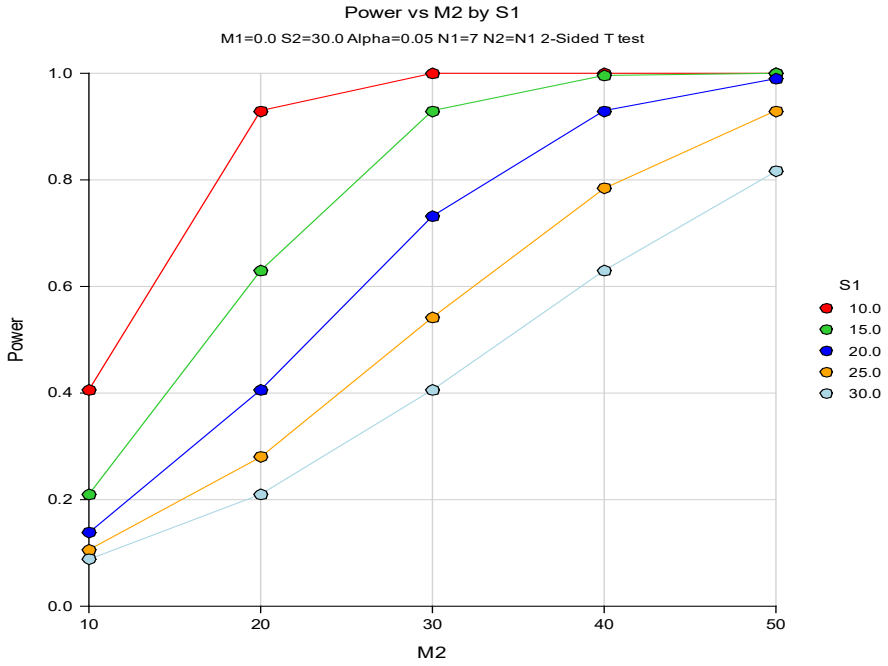


Table 11. Power estimates by mean difference in percent reduction in inflammation

Mean difference in percent reduction in inflammation	Power estimates for different standard deviations				
	SD 10	SD 15	SD 20	SD 25	SD 30
10%	40.6%	21.0%	X	X	X
20%	92.9%	63.0%	40.6%	28.1%	21.0%
30%	100%	92.9%	73.1%	54.1%	40.6%
40%	100%	99.5%	92.9%	78.5%	63.0%
50%	100%	100%	99.0%	92.9%	81.6%

With group sizes of 7, we would achieve at least 90% power in the primary efficacy endpoint analysis to detect a 30% difference between the groups in the percent reduction in inflammation if the standard deviation is 15 or less. Due to the relatively small sample size, all secondary endpoint analyses will be performed as descriptive analyses rather than statistical comparisons between groups.

16. Identification and Access to Source Data

16.1. Source Data

Source documents and source data are considered to be the original documentation where subject information, visits consultations, examinations and other information are recorded. Documentation of source data is necessary for the reconstruction, evaluation and validation of clinical findings, observations and other activities during a clinical trial.

16.2. Access to Source Data

The site investigators and site staff will make all source data available to the DAIT/NIAID, as well as to relevant health authorities. Authorized representatives as noted above are bound to maintain the strict confidentiality of medical and research information that may be linked to identified individuals.

17. Protocol Deviations

17.1. Protocol Deviation Definitions

Protocol Deviation – The investigators and site staff will conduct the study in accordance to the protocol; no deviations from the protocol are permitted. Any change, divergence, or departure from the study design or procedures constitutes a protocol deviation. As a result of any deviation, corrective actions will be developed by the site and implemented promptly.

Major Protocol Deviation - A Protocol Deviation is a variance from the IRB approved protocol that may affect the subject's rights, safety, or well-being and/or the completeness, accuracy and reliability of the study data. In addition, protocol deviations include willful or knowing breaches of human subject protection regulations, or policies, any action that is inconsistent with the NIH Human Research Protection Program's research, medical, and ethical principles, and a serious or continuing noncompliance with federal, state, local or institutional human subject protection regulations, policies, or procedures.

Non-Major Protocol Deviation - A non-major protocol deviation is any change, divergence, or departure from the study design or procedures of a research protocol that does not have a major impact on the subject's rights, safety or well-being, or the completeness, accuracy and reliability of the study data.

17.2. Reporting and Managing Protocol Deviations

The study site principal investigator has the responsibility to identify, document and report protocol deviations as directed by the study Sponsor. However, protocol deviations may also be identified during site monitoring visits or during other forms of study conduct review.

Upon determination that a protocol deviation has occurred, the study staff will complete a Protocol Deviation form. Protocol deviation reports will be compiled and reviewed by the NIAID/DAIT and the DSMB. Sites will be responsible for reporting deviations to local IRB's, as per local requirements.

18. Ethical Considerations and Compliance with Good Clinical Practice

18.1. Statement of Compliance

This clinical study will be conducted using good clinical practice (GCP), as delineated in *Guidance for Industry: E6 Good Clinical Practice Consolidated Guidance*, and according to the criteria specified in this study protocol. Before study initiation, the protocol and the informed consent documents will be reviewed and approved by each site *IRB*. Any amendments to the protocol or to the consent materials will also be approved by the respective IRB before they are implemented.

18.2. Informed Consent Process

The consent process will provide information about the study to a prospective participant and will allow adequate time for review and discussion prior to his/her decision. The principal investigator or designee listed on the FDA 1572 will review the consent and answer questions. The prospective participant will be told that being in the trial is voluntary and that he or she may withdraw from the study at any time, for any reason. All participants (or their legally acceptable representative) will read, sign, and date a consent form before undergoing any study procedures. Consent materials will be presented in participants' primary language. A copy of the signed consent form will be given to the participant.

The consent process will be ongoing. The consent form will be revised when important new safety information is available, the protocol is amended, and/or new information becomes available that may affect participation in the study.

18.3. Privacy and Confidentiality

A participant's privacy and confidentiality will be respected throughout the study. Each participant will be assigned a unique identification number and these numbers rather than names will be used to collect, store, and report participant information. Site personnel will not transmit documents containing personal health identifiers (PHI) to the study sponsor or their representatives.

19. Publication Policy

The CTOT policy on the publication of study results will apply to this trial.

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Appendix 1. Donor Assessments

	Screening
Visit Number	D1
Study Assessments (All Donors)	
Recipient Eligibility and Consent	x
Demographics (Age at Donation, Gender, Ethnicity)	x
Donor HLA Typing (retrospective chart review)	x
EBV and CMV IgG[1]	x
HBV cAb, HBV sAg, HBV sAb quantitative[1]	x
Limited Medical History	x

[1] HLA Typing and viral serologies performed as part of evaluation for donation can be collected retrospectively.

Appendix 2. Group 1 Recipient Schedule of Events

	Study Eligibility	Randomization	Day 41	Day 48	Day 55	Day 69	Day 125	Day 223	Day 405	Clinically Indicated Biopsy
Visit Number	Screen 1	Day 0	T0-G1	1	2	3	4	5	6	CIB
Visit Window	± 2 weeks of 6 month biopsy ǂ	≤ 2 weeks after Qualifying Biopsy	± 3 days	± 5 days				± 14 days		- 14 days prior to last study visit
General Study Assessments										
Study Eligibility ǂ	x									
Informed Consent	x									
Randomization		x								
Demographics, Transplant History	x									
Medical History	→	→		→	→	→	→	→	→	→
Physical Examination/ Vital Signs	x			x	x	x	x	x	x	
Review/Collect Concomitant Medications	→	→		→	→	→	→	→	→	→
Adverse Event/Serious Adverse Event Assessment	→	→		→	→	→	→	→	→	→
Local Laboratory Assessments										
Recipient HLA Typing[1]	x									
Donor Specific Anti-HLA Antibodies Class 1 and 2[1]	x						x	x	x	
CMV, EBV, HIV1, HIV2, HBV (HBsAg, HBcAb), HCV (HCVAb) serology[1]	x									
CMV, EBV by PCR	x			x	x	x	x	x	x	
BKV by PCR in serum	x						x	x	x	
HCV PCR (for subjects with positive HCVAb)	x									
SARS-COV2 RT-PCR	x[4]									
CBC (with differential and platelets)	x		x	x	x	x	x	x	x	x
CD4 Count	x				x	x	x	x	x	x
PT/INR	x							x[2]		x
Basic Chemistry (Na, K, Cl, CO_2, BUN, Glucose, Creatinine)	x		x	x	x	x	x	x	x	x
Liver Tests (ALT, AST, T Bilirubin, D Bilirubin)	x		x	x			x	x	x	
Tacrolimus Level	x		x	x	x	x	x	x	x	x[3]
Fasting Lipid Panel	x						x	x	x	
Urine Protein and Creatinine Ratio	x		x	x	x	x	x	x	x	
Urine Pregnancy Test	x									
Local Pathology Results - Graft Histology (Standard of Care Biopsy)	x							x		x
Central Laboratory Assessments										
Blood Specimens										
TruCount[3] (UCSFa)	x									
MFC Panels (Percentage Tregs/ Leukocyte phenotypes in Blood) - PBMC (UCSFb)					x	x	x	x	x	x
kSORT and kSPOT (Gene Expression in Blood) - Paxgene Tube (UCSFb)	x				x	x	x	x	x	
non- HLA Alloantibodies in Blood - 5 ml Red Top Tube (UCSFb) Fibrosis Biomarkers in Serum - Serum from Red Top Tube (UAB)	x				x		x	x	x	x
Renal Biopsy Specimens										
mIF/ISH (Intragraft Cytokine Expression) - 1/2 16g Core Formalin Fixed Paraffin-Embedded (UCSFc)	x							x		x
CRM (Transcriptional Profiling of Graft) - performed with FFPE tissue (UCSFc)	x[2]							x		x
Pathology Slides (biopsy case) for Central Pathology Reading (UCSFc)	x							x		x
Urine Specimens										
Protein Biomarkers and RNA in Urine (Shared 100ml Urine Collection (Pellet & Supernatant to UCSFb) Fibrosis Biomarkers in Urine (Shared 100ml Urine Collection (Supernatant to UAB)	x			x	x	x	x	x	x	x

ǂ Screening biopsy date must be 5 months after transplant ± 8 weeks. Except as marked, all screening labs should be dated ± 2 weeks of biopsy date, keeping in mind lab results must be available within window for randomization.

[1] HLA Typing, DSA, and viral serologies performed as part of evaluation for transplantation can be collected retrospectively. If not available, serologic testing should be done within 2 weeks of screening biopsy. EBV, should be repeated within screening window if previously negative.

[2] PT/INR should be collected as part of standard of care assessment prior to biopsy.

[3] TruCount will only be performed at the screening visit.

[4] SARS-COV2 testing does not have to be repeated if negative at time of biopsy.

Appendix 3. Group 2 Recipient Schedule of Events

Visit Label/ Days after Treg Infusion	Study Eligibility	Randomization	PBMC Collection Group 2	Treg Infusion	Day 1	Day 7	Day 14	Day 28	Day 84	Day 182	Day 364	Clinically Indicated Biopsy
Visit Number	Screen 1		T -16	T0	T1	1	2	3	4	5	6	CIB
Visit Window	± 2 weeks of 6 month biopsy ǂ	≤ 2 weeks after Qualifying Biopsy	Infusion can occur any time up to and including day 62 after central pathology confirmation.		± 1 hr	± 1 day	± 3 days	± 3 days		± 14 days		- 14 days prior to last study visit
General Study Assessments												
Study Eligibility ǂ, Treg Eligibility, mTOR Eligibility	x			x				x				
Informed Consent	x											
Randomization		x										
Demographics, Transplant History	x											
Medical History	→	→	→	→	→	→	→	→	→	→	→	→
Physical Examination/ Vital Signs	x		x			x	x	x	x	x	x	
Review/Collect Concomitant Medications	→	→	→	→	→	→	→	→	→	→	→	→
Adverse Event/Serious Adverse Event Assessment	→	→	→			→	→	→	→	→	→	→
Local Laboratory Assessments												
Recipient HLA Typing[1]	x											
Donor Specific Anti-HLA Antibodies Class 1 and 2[1]	x								x	x	x	
CMV, EBV, HIV1, HIV2, HBV (HBsAg, HBcAb), HCV (HCVAb) serology[1]	x											
CMV, EBV by PCR	x					x	x	x	x	x	x	
BKV by PCR in serum	x								x	x	x	
HCV PCR (for subjects with positive HCVAb)	x											
SARS-COV2 RT-PCR	x[6]			x[6]								
CBC (with differential and platelets)	x[5]		x[5]	x	x	x	x	x	x	x	x	x
CD4 Count	x				x	x	x	x	x	x	x	x
PT/INR	x						x			x		x
Basic Chemistry (Na, K, Cl, CO_2, BUN, Glucose, Creatinine)	x			x	x	x	x	x	x	x	x	x
Liver Tests (ALT, AST, T Bilirubin, D Bilirubin)	x			x		x		x	x	x	x	
Tacrolimus Level	x			x	x	x	x	x[2]	x[2]	x[2]	x[2]	x[2]
Everolimus Level *(Group 2 subjects who convert only)*								x[2]	x[2]	x[2]	x[2]	x[2]
Fasting Lipid Panel	x								x	x	x	
Urine Protein and Creatinine Ratio	x			x	x	x	x	x	x	x	x	
Urine Pregnancy Test	x			x								
Local Pathology Results - Graft Histology (Standard of Care Biopsy)	x									x		x
Central Laboratory Assessments												
Manufacturing												
Blood Collection (450-500ml) or Leukapheresis for PBMC Isolation/ Treg Manufacturing (UCSFa)			x									
Blood Specimens												
Deu-bl (Detection of Deuterated Cells in Blood) - PBMC (UCSFa) TruCount[4] (UCSFa) MFC Panels (Percentage Tregs/ Leukocyte phenotypes in Blood) - PBMC (UCSFb)	x[4]				x	x	x	x	x	x	x	x
kSORT and kSPOT (Gene Expression in Blood) - Paxgene Tube (UCSFb)	x						x	x	x	x	x	
non- HLA Alloantibodies in Blood - 5 ml Red Top Tube (UCSFb) Fibrosis Biomarkers in Serum - Serum from Red Top Tube (UAB)	x						x		x	x	x	x
Plasma for Banking (6 ml Lavender EDTA Tube (UCSFb)				x								
Serum for Banking (4 ml Red Top Tube (UCSFb)				x								
Renal Biopsy Specimens												
Deu-biopsy (Detection of Deuterated Cells in Graft) - 1/2 to 1 16g core in PBS (UCSFa)							x					
Central Pathology Reading - shared specimen with mIF/ISH FFPE (UCSFa)							x					
mIF/ISH (Intragraft Cytokine Expression) - 1/2 16g Core Formalin Fixed Paraffin-Embedded (UCSFc)	x						x			x[3]		x[3]
CRM (Transcriptional Profiling of Graft) - performed with FFPE tissue (UCSFc)	x						x			x		x
Pathology Slides (biopsy case) for Central Pathology Reading (UCSFc)	x									x		x
Urine Specimens												
Protein Biomarkers and RNA in Urine (Shared 100ml Urine Collection (Pellet & Supernatant to UCSFb) Fibrosis Biomarkers in Urine (Shared 100ml Urine Collection (Supernatant to UAB)	x			x		x	x	x	x	x	x	x

ǂ Entry into the study is based on results of a post-transplant standard of care surveillance biopsy. This biopsy must be obtained at 5 months (+/- 8 weeks) from the day of transplantation. Screen 1 labs must be drawn ± 2 weeks from the date of the biopsy, keeping in mind lab results must be available within window for randomization. Study group allocation must take place no more than 2 weeks after the central pathologist confirms eligibility.

[1] HLA Typing, DSA, and viral serologies performed as part of evaluation for transplantation can be collected retrospectively. If not available, serologic testing should be done within 2 weeks of screening biopsy. EBV must be repeated within screening window if previously negative.

[2] Collect relevant trough level only, not necessarily both. EVR trough levels should be obtained at least weekly until target level is reached/maintained.

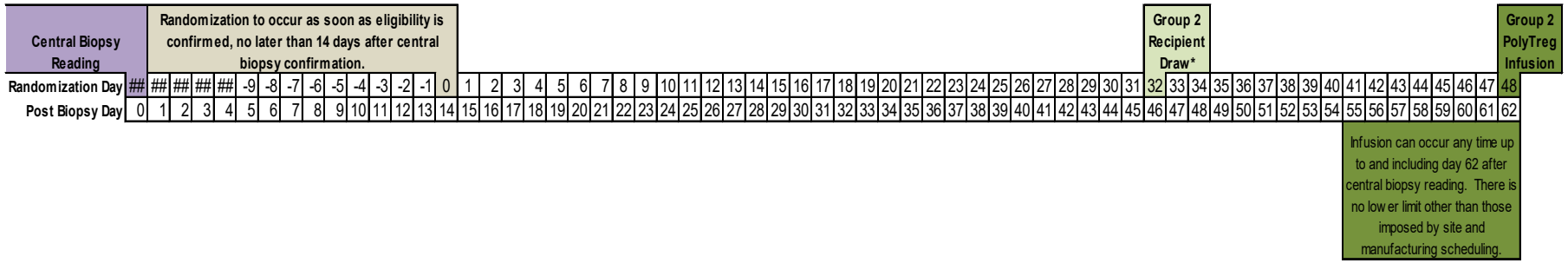
[3] If inflammation is detected in 6 month biopsies, deuterium detection may be performed on histological sections.

[4] TruCount will only be performed at the screening visit.

[5] CBC at screening should be utilized for eligibility and determination of PBMC collection method. CBC should be repeated prior to PBMC collection.

[6] SARS-COV2 testing does not have to be repeated if negative at time of biopsy. SARS-COV2 testing must be done within 1 week of polyTreg infusion.

Appendix 4. Manufacturing Timeline



*Group 2 recipient draw must occur 16 days (14-16 days for UCSF) prior to infusion date.

Amendment 8: Group 1 SOE will maintain Day 0 as day of study group assignment. Day 0 label was removed from the Group 2 and 3 SOE to avoid confusion. For Group 2 and 3, the day of infusion will no longer be linked to study group assignment but rather to central biopsy reading confirmation.